Studies on the enzyme glucose oxidase from

Penicillium amagasakiense

H.Geisow.

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SUMMARY

A short method of purification of P.amagasakiense glucose oxidase has been achieved. Attempts to prepare the apoenzyme were unsuccessful. Inhibition of activity of the holoenzyme by heavy metals was investigated. It was shown that inhibition was due to the metal cations rather than the undissociated metal salts.

The effect of bisulphite on the enzyme was shown to be the same as for Aspergillus niger glucose oxidase. From the kinetic experiments with bisulphite, an ionizable group with a pK of 4.2 was found to be involved in the reaction. This pK was assigned to the amino group of adenine of the FAD moiety of the enzyme. Experiments on the binding of halide anions to the enzyme indicated the involvement of an ionizable group with the same pK.

Photo-chemical experiments on the enzyme revealed three different phenomena. (1) Photo-oxidation with methylene blue or Rose bengal as sensitiser, destroyed an ionizable group with pK of 7.2. This was assigned to a histidine residue in the protein. (2) At high pH in the presence of EDTA and with no oxygen present photo-reduction of the enzyme spectrum was obtained. The spectrum of the oxidised enzyme was obtained when air was admitted to the reduced species. (3) At high pH in the presence of EDTA, light at 450 nm wavelength caused the enzyme to lose activity. This activity loss was irreversible with respect to oxygen or glucose. The presence of glucose in the reaction mixture during illumination protected the enzyme from this photo-destruction.

INDEX OF CHAPTERS

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ABBREVIATIONS

The revised instructions to authors (1971) of the Biochemical Journal, published by the Biochemical Society, London, have been followed where ever possible. Recommended SI symbols for units have been used.

Table 1.1. A comparison of specific activities of various preparations of glucose oxidase.

Source	Assay conditions (see below)	Specific activity (μmol/min/mg dry weight)	Standardised activity, 25° glucose (μmol/min/mg dry weight).
A. niger (Franke and Deffner 1939)	Manometry, 30° excess glucose	37.8+	35.0
P. notatum (Coulthard et al 1945)	Manometry, 30° 0.8 M-glucose	40.2	37.8
P. notatum (Keilin and Hartree 1948a)	Manometry, 39° 0.05 M-glucose	73++	64
P. amagasakiense (Kusai 1960)	Manometry, 30° 0.12 M-glucose	110	104.5
P. amagasakiense (Nakamura and Ogura 1962)	Stopped flow, 25° excess glucose	77	77

All assays performed with phosphate or acetate buffer pH5.6, and excess catalase, except the experiments of Franke and Deffner, Coulthard et al, Nakamura and Ogura in which catalase was absent. Their values have been corrected for altered stoichiometry.

+ This preparation had a specific activity of 5.36 μmol/min/mg, but was considered only 5-10% pure by Keilin and Hartree.

++ This preparation had a specific activity of 61 μmol/min/mg, but was shown to be only 80-90% pure.

CHAPTER ONE HISTORICAL INTRODUCTION

The enzymology of gluconic acid formation was investigated by Muller (1926 - 1941) using the press juices from the mycelia of the fungi of A. niger and Penicillium glaucum. Slight purification of the enzyme was achieved by Franke and his co-workers Deffner and Lorenz (1937 - 1948), who also demonstrated that the activity of the different enzyme preparations was proportional to the flavin content, and that hydrogen peroxide was formed during the catalysis.

The systematic name which has been given to the enzyme is β-D glucose : O_2 oxidoreductase E.C.1.1.3.4. (Report of the Commission on enzymes, I.U.B. 1961). The trival name is glucose oxidase. Keilin and Hartree (1946, 1948a, b,) have shown that the enzyme has a prosthetic group of flavin-adenine-dinucleotide (FAD) and a small carbohydrate moiety comprising less than 20% of the total enzyme (Pazur, Kleppe and Cepure 1965). It catalyses an overall 2-electron oxidation-reduction reaction between β-D glucose and oxygen, as shown in equation 1.i. overleaf. The formation of hydrogen peroxide in the presence of glucose and oxygen gave the enzyme a reputation for antibiotic activity. Hydrogen peroxide has a bactericidal effect because of its vigorous oxidising power, and this led early workers to call their material Penicillin A (later notatin), penatin or Penicillin B.

This type of enzyme has only been isolated definitely from fungi. Enzyme from four fungal sources has been identified and purified :-

from Penicillium notatum	Coulthard et al.,1942,1945
from Penicillium amagasakiense	Kusai et al, 1960, Kusai 1960.
from Aspergillus niger	Swoboda et al., 1963, Pazur and Kleppe 1964
from Penicillium vitale	Pidoplichko et al 1965

and their activities compared in table 1.1.

Several workers have carried out an extensive screening programme of fungal species for glucose oxidase activity e.g. Gancedo 1967 and Grigorov 1969. The latter has shown that glucose oxidase activity can be obtained from all strains of Aspergilleae, depending on the time of incubation and carbohydrate growth source. Other glucose oxidases have been reported from non-fungal sources, but none of them has been purified and characterised as a flavoprotein oxidoreductase. (See the review of Schepartz and Subers 1964).

Muller claimed (1941) that A. niger possessed a glucose dehydrogenase as well as an oxidase. This dehydrogenase could not use oxygen as the electron acceptor, and the best acceptor was found to be 2,6-dichlorophenol-indophenol. Glucose dehydrogenase has been isolated from A. oryzae by Ogura (1939 - 1952). Kusai (1960) confirmed its presence in A. niger and showed that it was not a flavoprotein.

Considerable interest has been focussed on the use of glucose oxidase in the food industry and in clinical work. In 1949 a patent was issued "for the removal of oxygen from sealed food containers by glucose oxidase" (Sarett 1949). In medicine it is used to detect and measure quantitatively glucose in blood and urine samples, after the reported use in this field by Keston (1956).

Originally the assay for glucose oxidase activity utilised the hydrogen peroxide formed by the reaction, which was reduced to ~~oxygen and~~ water by peroxidase. This was coupled with the oxidation of a suitable chromagen (see equations 1. i. and 1. iii.). Such chromagens included o-tolidine, benzidine and o-dianisidine but because of the carcinogenic nature of these chromagens a new method has been evolved. The hydrogen peroxide is used as an oxidising agent for 4-amino phenazone in phenol (Trinder 1969)

glucose + oxygen $\xrightarrow{\text{glucose oxidase}}$ glucono-δ-lactone + H_2O_2 (i)

$H_2O_2 \xrightarrow{\text{catalase}} \frac{1}{2} O_2 + H_2O$ (ii)

$H_2O_2 + AH_2 \xrightarrow{\text{peroxidase}} A + 2\ H_2O$ (iii)

Manometry used to be the prime method of following glucose oxidase catalysis. Catalase was present in the assay which generated oxygen by the breakdown of the hydrogen peroxide formed. The amount of oxygen produced was measured and related to the peroxide formed, and hence to the enzyme turnover. Catalase which contaminates glucose oxidase preparations, alters the stoichiometry of the reaction so that only one atom of oxygen is used per molecule of glucose oxidised, (see 1.i. and 1.ii.). It is possible to use millimolar concentrations of cyanide in the assay which inhibit any traces of catalase present in the enzyme preparation. This restores the stoichiometry of the reaction to one molecule oxygen used per molecule glucose oxidised.

Oxygen uptake is now measured polarographically using a Clark-type oxygen electrode suitably connected to a pen recorder through a resistance box. It is also possible to follow hydrogen peroxide production spectrophotometrically at 235 nm in the presence of cyanide (Bright and Gibson 1967), or by any of the colorimetric methods mentioned previously using 1 M-H_2SO_4 to inactivate the enzyme after the reaction has proceeded for a period of time (Savage 1951, White and Secor, 1957, Pazur and Kleppe 1964).

The overall mechanism of glucose oxidase from P. notatum has been investigated by Bentley and Neuberger (1949) using isotopes of oxygen. Their findings are summarised in equations 1.iv. and 1.v.

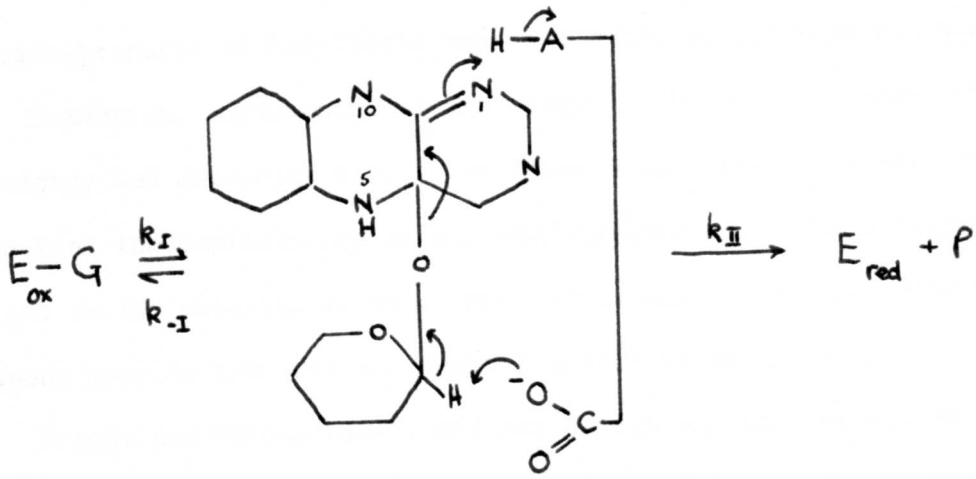
$$C_6H_{12}O_6 + O_2^{16} + H_2O^{18} \longrightarrow C_6H_{10}O_6 + H_2O_2^{16} + H_2O^{18} \quad \text{(iv)}$$

$$C_6H_{12}O_6 + O_2^{18} + H_2O^{16} \longrightarrow C_6H_{10}O_6 + H_2O_2^{18} + H_2O^{16} \quad \text{(v)}$$

Thus water is not involved in the reaction, the hydrogen peroxide being derived from the oxygen and the glucose. Moreover they found that the rate of gluconic acid formation was too slow to be part of the primary process which was the uptake of oxygen. The first product, the lactone, was detected polarographically and since it was found to be in the pyranose ring form, Bentley and Neuberger deduced that the glucose must be in the ring form too. The hydrolysis of the lactone to gluconic acid occurred spontaneously.

Keilin and Hartree (1952) showed that glucose oxidase was highly specific for the β-anomer of glucose, using P. notatum enzyme. This finding was confirmed for the enzyme from P. vitale (Degtyar, Gulii and Maizel 1965), A. niger (Pazur and Kleppe 1964) and P. amagasakiense (Kusai 1960). The substrate specificity of the latter enzyme species is shown in table 1.2. The effect of various substituents in different positions on the glucose ring has been studied by Pazur and Kleppe (1964). From this they concluded that either of the hydroxyl groups at positions 1 or 3, equatorial to the ring, might be the site at which the enzyme is attached to the glucose.

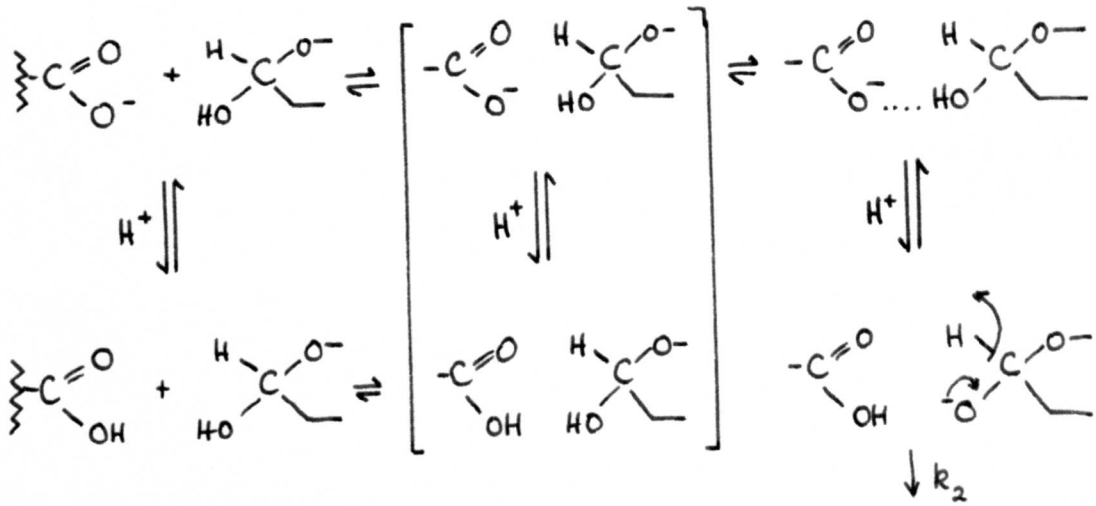
Earlier glucose oxidase was described as catalyst of an overall 2-electron oxidation-reduction reaction. Bright and Gibson (1967) used 1-deutero-glucose to investigate the hydrogen transfer from glucose to flavin. They found a kinetic isotope effect of between 10- and 15- fold associated with flavin reduction. This would be consistent with nucleophilic attack by the glucose C-1 hydroxyl group on the flavin nucleus, followed by proton abstraction from the carbon and electronic rearrangements (Weibel and Bright 1971). The kinetic isotope effect would come from the proton removal from glucose, controlled by k_2 in the mechanism overleaf



$$k_2 = \frac{k_I \, k_{II}}{k_{-I}}$$

The substrate attack is shown at position C-4a rather than the carbonyl group at C-5 suggested by some workers following the model studies of Brown and Hamilton (1970). Massey et al (1968) and Muller, Massey et al (1969) have shown that substrate attack on the oxidised flavin may occur at position C-4a or N-5 on the isoalloxazine ring. Weibel and Bright (1971) have identified an acidic amino-acid residue (pK_a = 10) close to the N-1 position represented by H-A in the above mechanism.

A second mechanism, which is consistent with the isotope effect involves hydride transfer from the carbon to the flavin (Weibel and Bright 1971).



Hydride transfer to the flavin occurs because of the electron sink effect.

Hemmerich, Nagelschneider and Veeger (1970) have reviewed the chemistry and molecular biology of flavins and flavoproteins. They discussed the implications of the two possible sites for substrate attack on the oxidised flavin. They discussed but did not distinguish between hydride transfer and group transfer to the flavin.

Bright and Gibson (1967) did not detect any isotope effect in the oxidative half reaction of glucose oxidase. This has led Weibel and Bright to suggest that the oxidation of flavin proceeds via electron transfer to oxygen followed by rapid proton dissociation from N-1 and N-5 of the isoalloxazine ring.

Most of the work on the glucose oxidase mechanism has been carried out on A. niger enzyme. It is hoped to show, in the following chapters, that the P. amagasakiense enzyme has the same mechanism.

CHAPTER TWO. THE PURIFICATION OF GLUCOSE OXIDASE FROM FUNGAL SOURCES.

Introduction

The first attempt at purification of glucose oxidase by Franke and Lorenz (1937) involved precipitating the enzyme from the press juices of A. niger mycelia. The mycelia were disrupted in a Buchner press at 300 atmospheres pressure. The enzyme was precipitated by the addition of a twelve-fold excess of a 2:1 mixture of alcohol and ether to the press juices. The specific activity of the preparation of Franke and Deffner (1939) at 30°C was 37 μmoles/min/mg dry weight.

Coulthard and co-workers (1945) using a culture filtrate of Penicillium notatum, concentrated it by acetone precipitation. Then they either precipitated the resuspended extract by addition of tannic acid, or Reinecke's salt to obtain a fairly pure product. Further purification was by acetone precipitation and ammonium sulphate fractionation at approximately 80% saturation. The specific activity of their preparation at 30°C was 40 μmol oxygen/min/mg dry weight. Using the same technique, Keilin and Hartree (1948) obtained a preparation with a specific activity of 73 (39°C).

Both Pazur and Kleppe (1964) and Swoboda and Massey (1965) have used a commercial A. niger mycelial preparation as their source of the enzyme. Pazur and Kleppe (1964) dialysed a solution of mycelial powder against water and using ammonium sulphate, obtained a fraction (between 60% and 87% ammonium sulphate saturation) which contained a high percentage of the original oxidase activity. This dialysed fraction was applied to a DEAE-cellulose column and eluted with 0.1 M-acetate buffer pH3.7, carbohydrate and catalase contaminants having been eluted earlier with 0.1 M-acetate buffer pH4.5. The yellow fraction was rechromatographed on a smaller column of DEAE-cellulose.

In contrast, Swoboda and Massey dialysed their extract against

Table 2.1. A comparison of the effectiveness of glucose oxidase purification by the method of Pazur and Kleppe (1964) and that of Swoboda and Massey (1965).

Method of Pazur and Kleppe

	Specific activity (units/ml)+	% recovery of total units
Enzyme sample	541	
After dialysis	186	93.76
$(NH_4)_2SO_4$ fractionation	680	60.71
1st DEAE-cellulose chromatography	766	-
2nd DEAE-cellulose chromatography	992	47.33

+ A unit was defined as causing the uptake of 11.2 µl oxygen/min at s.t.p. Activity measurements carried out at 30°C pH 5.9.

Method of Swoboda and Massey

	Specific activity+ µmol/min/mg protein	% recovery of total units
Enzyme sample	13.9	
After dialysis	28.0	100
Amberlite CG-50 chromatography	78.0	82.08
$(NH_4)_2SO_4$ precipitation and dialysis	82.0	77.25
DEAE-cellulose chromatography	92.0	46.90
$(NH_4)_2SO_4$ precipitation and dialysis	92.0	44.96

+ Activity measurements at pH 5.6, 37°C.

0.1 M-acetate buffer pH 4.5 and removed the precipitate formed during the dialysis by centrifugation. The supernatant was applied directly to a column of Amberlite-CG 50 (suitably treated). The column was washed with 0.1 M-buffer pH 4.5, and the enzyme was eluted with 0.1 M-acetate buffer pH 5.0. Protein was precipitated from the eluate by ammonium sulphate fractionation (80-90% saturation), redissolved, dialysed against 0.1 M-phosphate buffer pH 6.0 and applied to a DEAE-cellulose column. The enzyme was finally eluted by 0.2 M-phosphate buffer pH 6.0, to be followed by concentration with ammonium sulphate and dialysis. A table comparing the recovery of specific activity from the starting material and yield of total units in each of these two methods has been drawn up (table 2.1.).

A simple industrial purification of commercially available glucose oxidase giving a yield of 90% of initial activity relies on the precipitation of the enzyme by addition of methanol to an aqueous solution of the enzyme extract. A pH of 7.0 is continuously maintained. Hyflo-supercel is added and the precipitate is separated. The filter cake is homogenised in cold water pH 6.0, filtered and lyophilised. An enrichment of enzyme material of 300% is claimed possible (Bergmeyer et al 1969).

Purification of enzyme from P. vitale has been achieved by either putting the culture filtrate down a column of SG-I (equivalent to Amberlite-CG 50) and eluting with 0.6 M-acetate buffer pH 5.0 (Pokrovskaya and Chistyakova 1965) or by absorption of the enzyme from the culture fluid by anhydrous alumina pH 6.5-7.0 and elution by shaking with a 9% solution of ammonium chloride in 0.3 M-potassium dihydrogen phosphate (Gulii and Degtyar 1962). In each case the enzyme has been precipitated from the concentrated eluate by ethanol at -10^{o}.

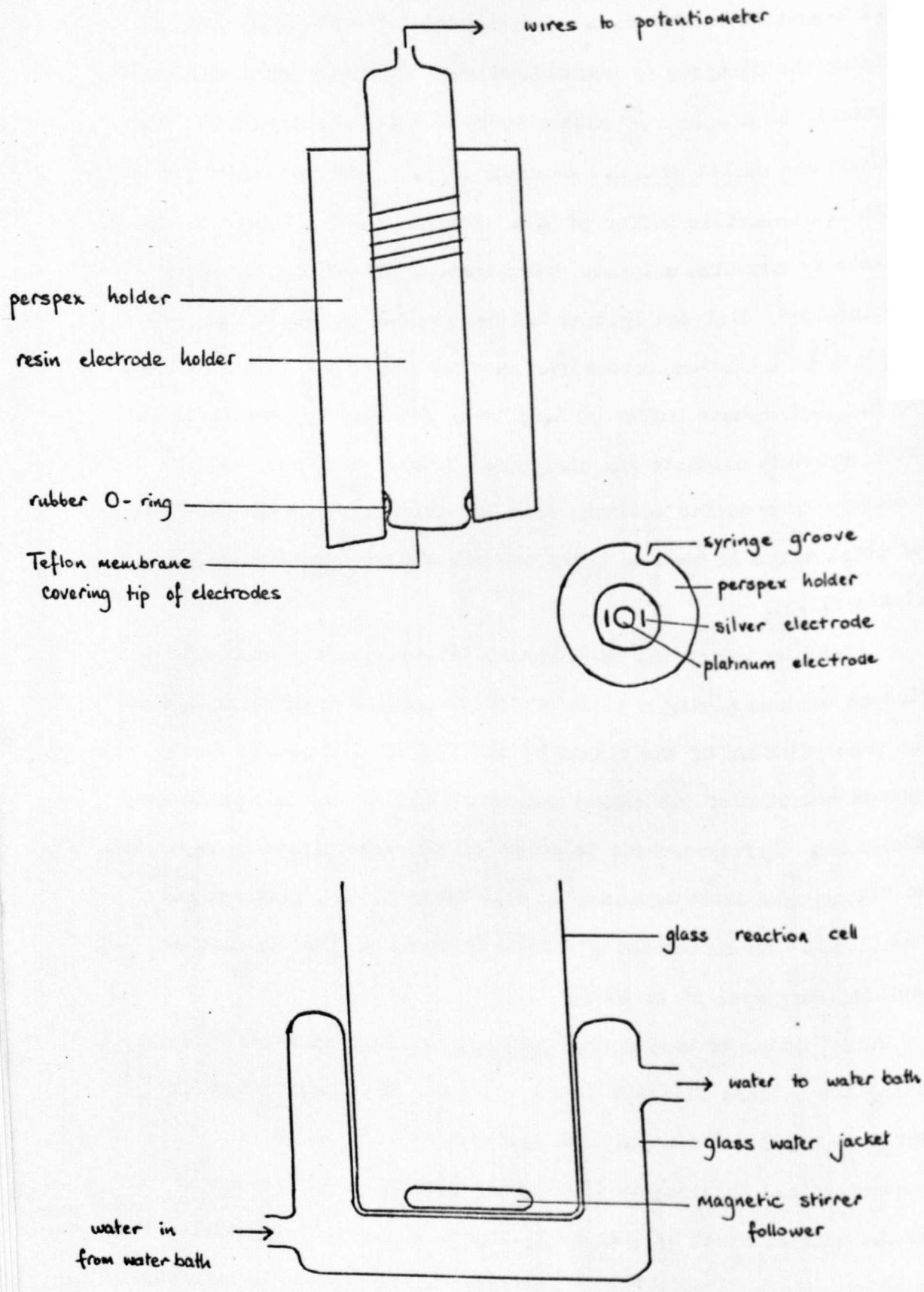


Fig.2.1. Clark type oxygen electrode used for measuring enzyme activity.

Only Kusai has published a purification procedure for Pencillium amagasakiense (Kusai 1960, Kusai et al 1960). He originally grew a culture of this fungus which he found to exude glucose oxidase in to the culture medium. A crude extract of this culture medium is now available commercially under the name "Deoxine" (Nagase and Co., Ltd.) Kusai dialysed the crude extract over-night against 0.1 M-acetate buffer pH 4.5, centrifuged to clear the solution and passed all the supernatant down an Amberlite-CG 50 column (5 x 25 cm) buffered with the same buffer. After washing with 2-3 l of buffer until the elute was clear, during which process half the glucose oxidase activity was eluted in the first washing, the rest of the enzyme was eluted with 0.1 M-acetate buffer pH 5.0. After fractionation and dialysis against 0.05 M-acetate buffer pH 5.0, the solution was passed through a second column of Amberlite-CG 50 (2 x 70 cm) equilibrated with the same buffer. The enzyme was eluted with the same buffer, was fractionated with ammonium sulphate, dialysed against water and crystallised with ammonium sulphate. The other glucose oxidase fraction from the first column would not crystallise on further purification and was thought by Kusai to contain partially modified enzyme.

Materials and Methods.

a). Method of measuring glucose oxidase activity.

Measurements of activity were made using a Clark type oxygen electrode as shown in fig.2,1. It consisted of a platinum electrode with a silver electrode on either side set in an inert resinous material. The tip of this assembly was covered by a thin Teflon membrane held in place by a rubber O-ring. Between the electrodes and the membrane was a drop of 0.1 M-KCl. The electrodes were connected

to an adjustable potentiometer box which was connected in turn to a pen recorder.

The electrode assembly was screwed into a solid perspex rod which just fitted a cylindrical glass electrode cell. The dimensions were so arranged that when the perspex electrode holder was fitted into the top of the cell, there was a space beneath the electrodes to contain exactly 5 ml. reaction mixture. Down the side of the perspex holder was a groove just large enough to allow a microsyringe needle to be introduced into the electrode cell.

The electrode cell was held in a glass water jacket connected to a thermostatted water bath and pump, and a temperature of 25°C was maintained. Additions of enzyme, substrate or inhibitor were made to the reaction mixture in the cell by means of a microsyringe used as a microburette (Scientific Glass Engineering, Melbourne, Australia) whose needle was passed down the groove in the electrode casing as described above.

For normal assays, unless otherwise stated, the assay mixture contained 0.01 M-glucose and 10^{-3} M-EDTA in 0.1 M-acetate buffer pH 5.6. During enzyme purification procedures, excess catalase (8 µg/ml, Sigma type C-100) was added because of the presence of contaminating catalase in small quantities. Using excess catalase, activity values for glucose oxidase were a half those obtained with no catalase present, because of the changed stoichiometry of the reaction (see equations 1.i. and 1.ii.).

The initial rate of oxygen uptake was obtained by measuring the initial gradient of the recorded curve of oxygen uptake against time, which was drawn out by the pen recorder. The units of specific activity used are µ.mol of oxygen/min/mg of protein. (Oxygen saturation of aqueous solutions in equilibrium with air at

25^{o} and atmospheric pressure is 2.6×10^{-4}M).

b) Method of estimating protein and enzyme concentration.

Protein absorbance was measured on a Unicam SP600 spectrophotometer (Unicam-Pye, Cambridge). 1 mg/ml (dry weight) protein has an optical density at 280 nm of 1.85 (Kusai 1960). During the preparation of the enzyme, protein was estimated by the biuret method (Gornall, Bardawill and David, 1949).

Enzyme concentration was calculated from the absorbance of the enzyme at 450 nm, using the millimolar extinction coefficient of 11.3 for active site concentration, and 22.6 for the millimolar concentration.

c) Treatment of DEAE-cellulose for making chromatography columns.

DEAE-cellulose was treated with 2 M-HCl, distilled water, 2 M-NaOH and finally distilled water to charge the cellulose. During these procedures, fines were removed at each stage. The charged material was suspended in 0.1 M-acetate buffer pH 5.6 (1.5 M-sodium acetate adjusted with 1.5 M-acetic acid to the required pH and diluted with distilled water). The material was packed into a column and equilibrated with 0.01 M-acetate buffer pH 5.6.

d) Treatment of dialysis tubing.

Before use all dialysis tubing was left in 5×10^{-4} neutralised EDTA for 30 min. and then washed thoroughly with distilled water.

e) Purification of glucose oxidase.

All steps were carried out in a cold room at 4^{o}C. Deoxine (Nagase and Co.Ltd.,Japan) was treated according to the method of Kusai et al (1960) to the end of the first chromatography step on Amberlite CG-50. Since the Deoxine powder as supplied was found to set like cement on addition of buffer, it was necessary to

leach out the yellow coloured material. This was done by making several additions of buffer, of about 5 ml, to the Deoxine powder which was ground up in a mortar and pestle. The volumes of yellow liquid were pooled and dialysed against the same buffer (0.1 M-acetate, pH 4.5) overnight, and chromatographed on Amberlite as described by Kusai. From this column all yellow fractions were collected and assayed for glucose oxidase activity and protein content, as described in a) and b) above. Active fractions were pooled and divided, to be treated in one of two ways:-

1. Continuing with the Kusai method of purification.
2. Adding ammonium sulphate to 80% saturation (at 4°C). The precipitate was taken up in 0.02 M-acetate buffer pH 5.6 and dialysed against distilled water overnight. The non-diffusible material was applied to a column of DEAE-cellulose (Whatman type 22) 30 x 4 cm which had been pre-equilibrated with 0.02 M-acetate buffer pH 5.6. The adsorbed enzyme material was washed with one litre of the same buffer and very slowly eluted with 0.05 M-acetate buffer pH 5.6.

Subsequent purifications of the enzyme were carried out using a simpler technique involving only one chromatographic step. The solution of yellow material from Deoxine powder was dialysed exhaustively against 0.01 M-acetate buffer pH 5.6 for 3 days. After centrifugation to clear the non-diffusible material, the supernatant was put onto a column of DEAE-cellulose (Whatman type 52) 20 x 5 cm, pre-equilibrated with the same buffer. After washing with 2-3 litres of buffer, the yellow band was eluted with 0.05 M-acetate buffer pH 5.6. 50-100 ml fractions were collected and assayed for enzyme activity and protein content. Active fractions were combined, and the total volume was reduced five-fold by pervaporation. That is, a length of visking tubing containing the solution was suspended in a forced draught of cold air (Sober 1965). The enzyme was precipitated

Table 2.2. A comparison of the purification methods applied to glucose oxidase from P.amagasakiense.

Using the Kusai method (Kusai et al 1960)	Specific activity (μmol/min/mg)	% recovery of total units
Exhaustive dialysis of 'Deoxine' in 0.1 M-acetate buffer pH 4.5	53	-
Chromatography on Amberlite CG-50, elution with 0.1 M-acetate buffer pH 5.0	61	82.5
$(NH_4)_2SO_4$ fractionation; dialysis against 0.1 M-acetate buffer pH 5.0	74	41
Amberlite chromatography, elution with 0.05 M-acetate buffer pH 5.0. $(NH_4)_2SO_4$ fractionation; dialysis against water	80	22.6

Using a modification of the Kusai method	Specific activity (μmol/min/mg)	% recovery of total units
Exhaustive dialysis of 'Deoxine' in 0.1 M-acetate buffer pH 4.5	53	-
Chromatography on Amberlite CG-50, elution with 0.1 M-acetate buffer pH 5.0	61	82.5
$(NH_4)_2SO_4$ fractionation; dialysis against water. Chromatography on DEAE-cellulose, elution with 0.05 M-acetate buffer pH 5.6	56	50.8
$(NH_4)_2SO_4$ fractionation and dialysis against water, twice.	64.2	20

At the chromatography steps a number of yellow fractions were collected and the specific activity above is the mean value of the fractions obtained.

from solution by addition of solid ammonium sulphate to 90% saturation and was dialysed against distilled water overnight.

Other methods of concentrating the column eluate were tried including absorption of water by Sephadex G-50 (Pharmacia, Sweden) and a simple form of ultrafiltration where a negative pressure was applied outside a visking tube containing the solution and supported by a nylon bag (Sober et al 1956, Sober et al 1965).

f) Criteria of purity of the sample.

The purity of the enzyme was investigated with respect to catalase. Assays of glucose oxidase activity were performed as described in a) above, with and without excess catalase being added. In addition assays were carried out with no added catalase, but 10^{-3} M-potassium cyanide present to inhibit any traces of contaminating catalase.

The enzyme was also examined spectrophotometrically between 300 and 550 nm using a Cary model-14 spectrophotometer. The record obtained was compared with that of Kusai (1960), when any chromophoric impurities would have been noticed. The specific activity of the final preparation was compared with that of Kusai (1960) who investigated and confirmed the homogeneity of his preparation by ultracentrifugal and electrophoretic methods.

Results.

Neither the Kusai method nor the modification of it gave a satisfactory purification of the enzyme. A table comparing the effectiveness of the two methods is given in table 2.2. For all subsequent purifications only the one using one chromatographic step on DEAE-cellulose was used. Adsorption onto and elution from an ion-exchange column (DEAE-cellulose) achieved considerable purification. When the dialysed fluid was passed down the column, the yellow

Table 2.3. Purification of glucose oxidase involving one chromatography step.

	Volume (ml)	Protein (mg/ml)	Specific activity (μmol/mg/min)	Total activity (μmol/min)
Dialysis of enzyme material from 10 g 'Deoxine' against 0.01 M-acetate buffer pH 5.6 for 3 days	110	1.4	45.0	6878
Material chromatographed on DEAE-cellulose, washed with 0.01 M-acetate buffer pH 5.6, eluted with 0.08 M-acetate buffer pH 5.6	290	0.16	60.0	2784
Pervaporation	55	0.83	60.0	2739
$(NH_4)_2SO_4$ precipitation, overnight dialysis against distilled water.	2.9	7.0	65.0	1320

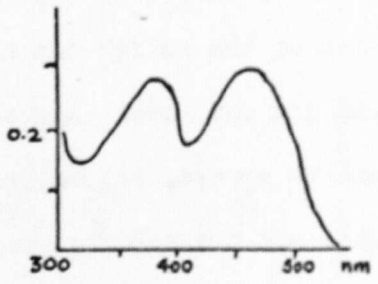
In this purification the recovery of total units was 20% compared with a yield of 36% by Kusai (1960)

component formed a discrete band at the top of the column. No yellow fraction was lost during the washing process; indeed until the strength of the buffer was increased from 0.01 M to 0.03 M the yellow band did not move. Addition of the 0.03 M-buffer caused the yellow band to spread, but 0.05 M kept the band more or less intact as it eluted off the column. The use of 0.08 M-buffer ensured that the yellow band eluted in a minimum volume. If much stronger buffer solutions were used some of the yellow band streaked down the column ahead of the main band (possibly analogous to the partially modified enzyme of Kusai.)

As the yellow band eluted a wide dark brown band was left at the top of the column. It was seen to move only very slowly under the eluting conditions described, and was thought to be the major concentration of catalase, the major contaminant of Deoxine and other sources of glucose oxidase. Swoboda (1963) noted that two peaks having catalase activity were obtained in his final elution off the DEAE column. The first eluted with 0.1 M-phosphate buffer pH 6.0, the second peak eluted with 0.2 M-phosphate buffer pH 6.0 at the front of the activity peak of glucose oxidase.

The recovery of total activity and enhancement of specific activity at each step are shown in table 2.3. for an average preparation.

The protein content of the yellow fractions was rather low (0.1-1.0 mg/ml protein) and contained in large volumes of eluting buffer. Ammonium sulphate fractionation is inefficient when very dilute protein solutions are used. Therefore attempts were made to reduce the volume. Surrounding the solution in visking tubing with Sephadex was effective but expensive and slow (Sephadex takes up 2-5 g water/g dry weight). There was also the possibility of ultra-violet absorbing impurities diffusing from the Sephadex into the solution (R.Brown personal communication). Practical difficulties with the ultra-filtration



Spectrum from Kusai (1960)

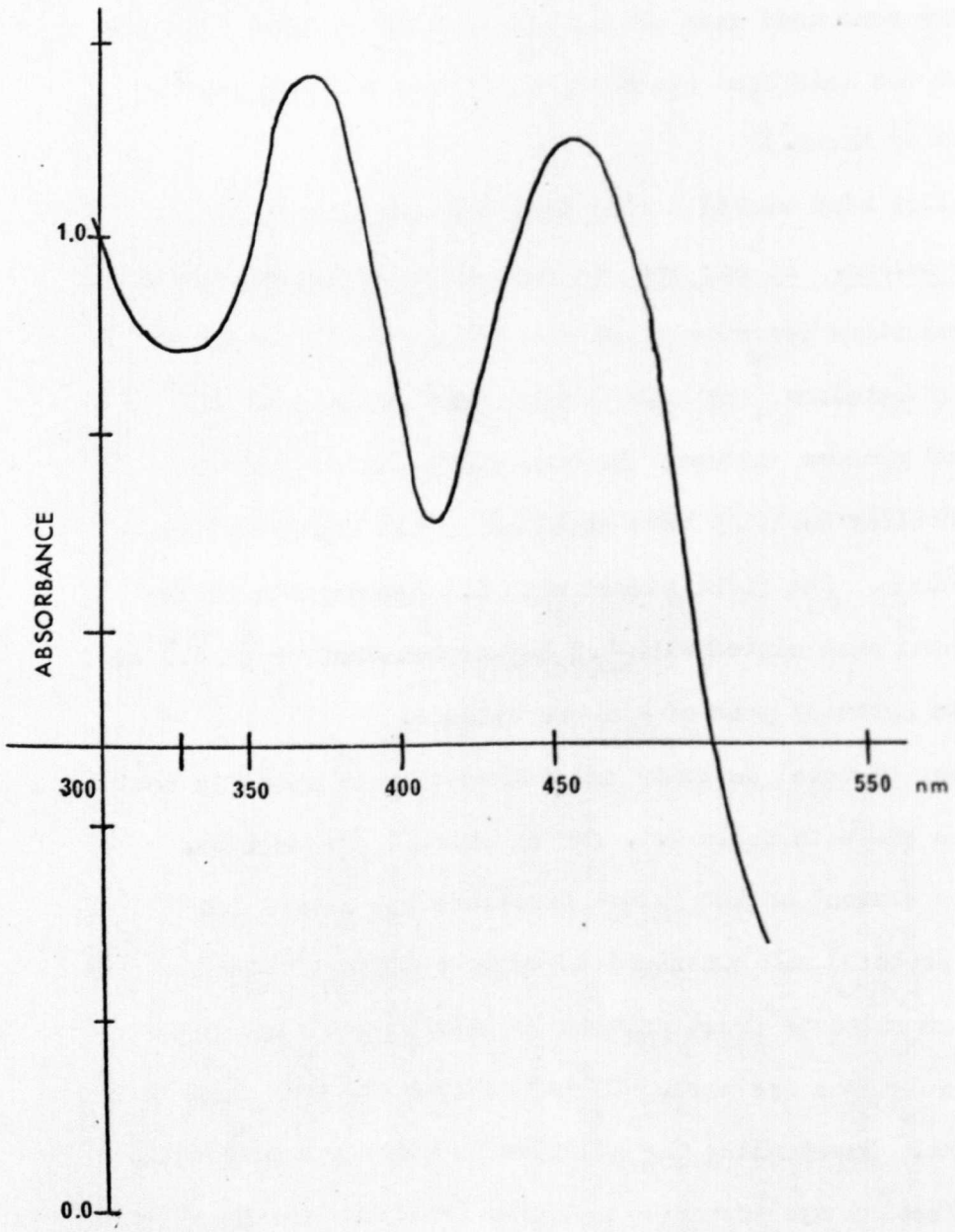


Fig.2.2. Comparison of the spectra of glucose oxidase from P.amagasakiense.

technique led to the pervaporation method being used. A five-fold reduction in volume was obtained.

The enzyme solution was concentrated further by ammonium sulphate precipitation. All the protein and enzyme activity were found in the precipitate at 90% saturation. No precipitate was obtained at lower saturation. Hence this step was one of concentration rather than purification.

The results of a typical assay with and without catalase in excess, and with potassium cyanide are given in table 2.4. The action of catalase on one of the products, hydrogen peroxide, is such that the stoichiometry of the glucose oxidase reaction is changed by the order of two (see equations 1.i and 1.ii). For each molecule of oxygen taken up, half a molecule of oxygen is released as a result of the catalase action. Thus for a catalase-free enzyme preparation the assay in the presence of excess catalase should have half the activity of that in the absence of catalase. Similarly, since potassium cyanide is a well-known inhibitor of catalase, then in the presence of excess cyanide an assay of catalase-contaminated enzyme will show an increased activity over that in the absence of cyanide. Catalase-free glucose oxidase is unaffected by potassium cyanide.

From table 2.4. it can be seen that, within experimental error, the sample was essentially catalase-free.

Table 2.4. Assay of purified glucose oxidase, pH 5.6

	Specific activity μ mol/min/mg		
	Experimental values		Mean
Assay of enzyme alone	178.5,	179.1	178.8
" " " with excess catalase	85.84,	93.85	89.84
" " " with 10^{-3}M-KCN	179.1,	183.7	181.4

A comparison of the spectrum of purified enzyme with the spectrum of that prepared by Kusai from his paper (Kusai 1960) is made in fig.2.2.

Discussion.

The purity of an enzyme is established by showing that the final product of purification consists of only one protein. If the product appears homogenous by several different tests then there is a high probability that it is the pure enzyme. Most of the tests are based of the physical properties of proteins, but they may include tests on catalytic properties also. Kusai et al (1960) established the purity of their glucose oxidase preparation in the following ways;-

1. By measuring spectrophotometrically the absorbance at 280nm per absorbance at 460 nm. This ratio measured the protein content of the preparation per flavin moiety, and was found to be constamt for the final fractionation, crystallisation and recrystallisation. Similarly lack of contamination by coloured impurities was measured by the ratio of absorbance at 380 to 460 nm.
2. Ultracentrifugation was carried out in 0.1 M-acetate buffer pH 5.6, and from the pattern obtained the enzyme was said to be homogenous.
3. Electrophoresis in 0.1 M-acetate buffer pH 4.85 again showed the enzyme to be homogenous.

None of these tests was carried out for the present enzyme preparations. During chromatography it was noted that as the discrete yellow band with glucose oxidase activity was eluted from the column, a brown coloured material possibly catalase, remained at the top. Assays of the final product with excess catalase or with 10^{-3} M-KCN (table 2.4.) showed that the enzyme was not seriously contaminated with catalase. The spectrum of this enzyme was very similar to that of Kusai's preparation. The latter had a specific activity of of 100 mol oxygen uptake/mg protein/min compared with an average value of 65 for this preparation.

Unfortunately enzyme purity was not fully established and final specific activity was low. Considerable loss of activity occurred at the chromatography stage and further work should have been done in this area if this shorter purification method is to supersede that of Kusai.

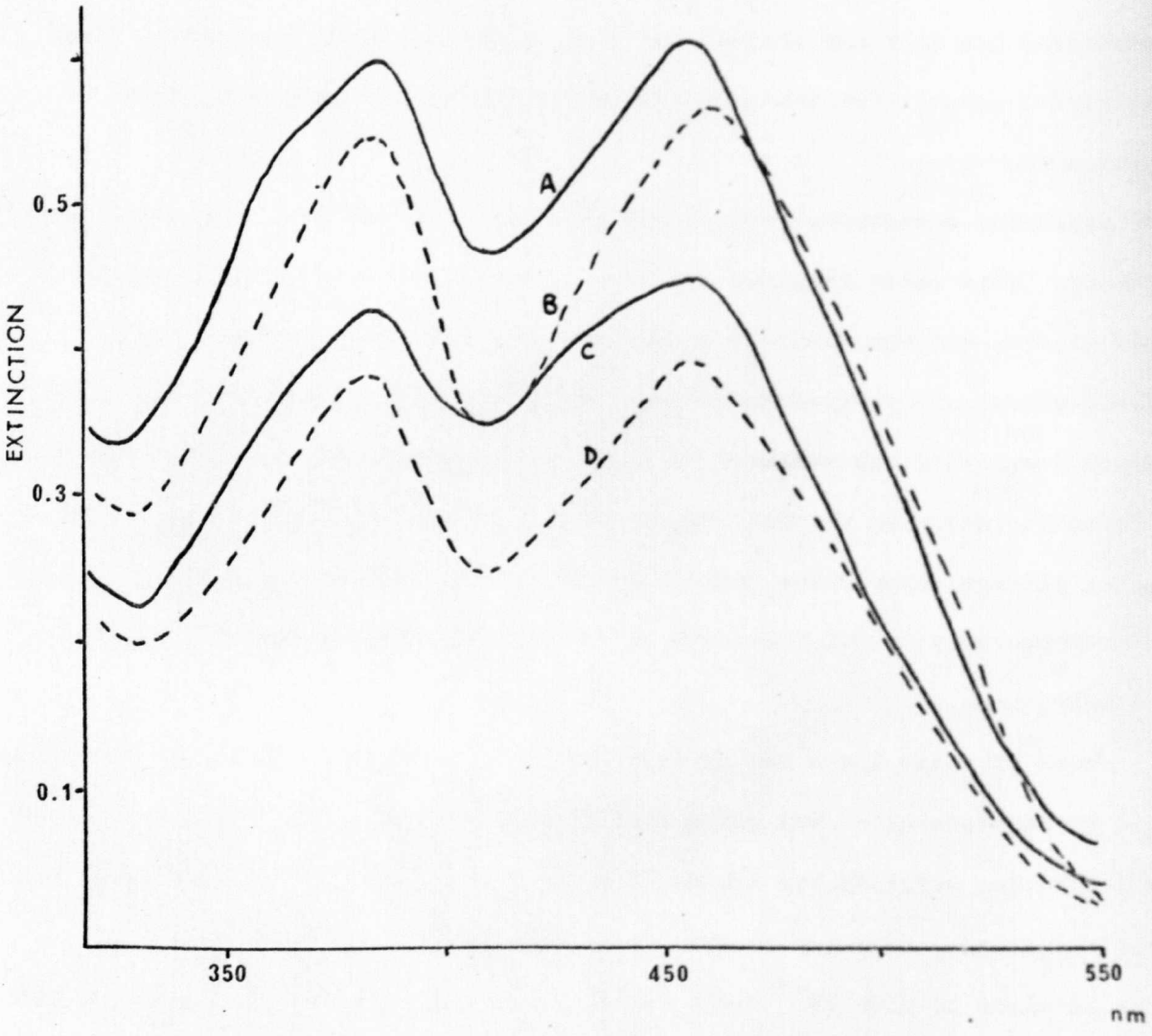


Fig.3.1. Absorption spectrum of reconstituted enzyme, from apoenzyme and FMN or FAD. (Kusai 1960)

Curve A FMN-enzyme, oxidised form.

Curve C " " reduced with glucose.

Curve B FAD-enzyme, oxidised form.

Curve D " " reduced with glucose.

CHAPTER THREE — THE ATTEMPTED PREPARATION OF THE APOENZYME OF P. AMAGASAKIENSE GLUCOSE OXIDASE AND RECOMBINATION EXPERIMENTS WITH THE APOENZYME OF A. NIGER GLUCOSE OXIDASE.

Introduction.

Kusai (1960) described the preparation of the apoprotein of P. amagasakiense glucose oxidase, by an acid-ammonium sulphate method at 0°. He was able to recombine the apoprotein with both FAD and FMN and obtained restoration of 93% initial activity in the former case. FMN did not reactivate the enzyme, but it was found to combine with the apoprotein. Addition of glucose to both the FAD-enzyme and the FMN-enzyme brought about reduction of the prosthetic group and a change in their absorption spectra (see fig.3.1.). However the results showed that the newly synthesised enzyme contained 6 moles flavin per mole enzyme, of which 2 moles flavin were reduced by glucose addition. He did not obtain enhanced flavin content when native enzyme was incubated with excess FAD. To account for these facts, it was suggested that the protein was probably modified by the acid-ammonium sulphate treatment, enabling a maximum combination of 6 moles flavin per mole enzyme. But further experiments failed to confirm that the protein moiety had been modified sufficiently to be more susceptible to proteinase digestion.

Although acid-ammonium sulphate treatment is most commonly used to split this flavoprotein enzyme into its component parts (see Pazur and Kleppe 1964, Swoboda 1969a) it has been reported that ultra-violet light will elicit dissociation of the FAD- protein complex of the P. vitale enzyme (Levina et al 1965). No attempt was made by these workers to reactivate the enzyme. Klarner and co-workers (1969) have demonstrated the dissociation of part of their sample of glucose oxidase from A. niger, into flavin and apoprotein after dialysis against 0.02 M-phosphate buffer pH 11.4. They claim that full activity was regained on neutralisation of the solution containing both parts of the enzyme.

Dialysis against 1 M-potassium bromide has effected the dissociation of the flavoprotein enzyme D-amino acid oxidase (Massey and Curti 1966), while Komai, Massey and Palmer (1969) have obtained deflavo xanthine oxidase by treatment of the holoenzyme with calcium chloride at high concentration, when the flavin was released as FMN. (High concentrations of calcium or magnesium ions cause hydrolysis of FAD.)

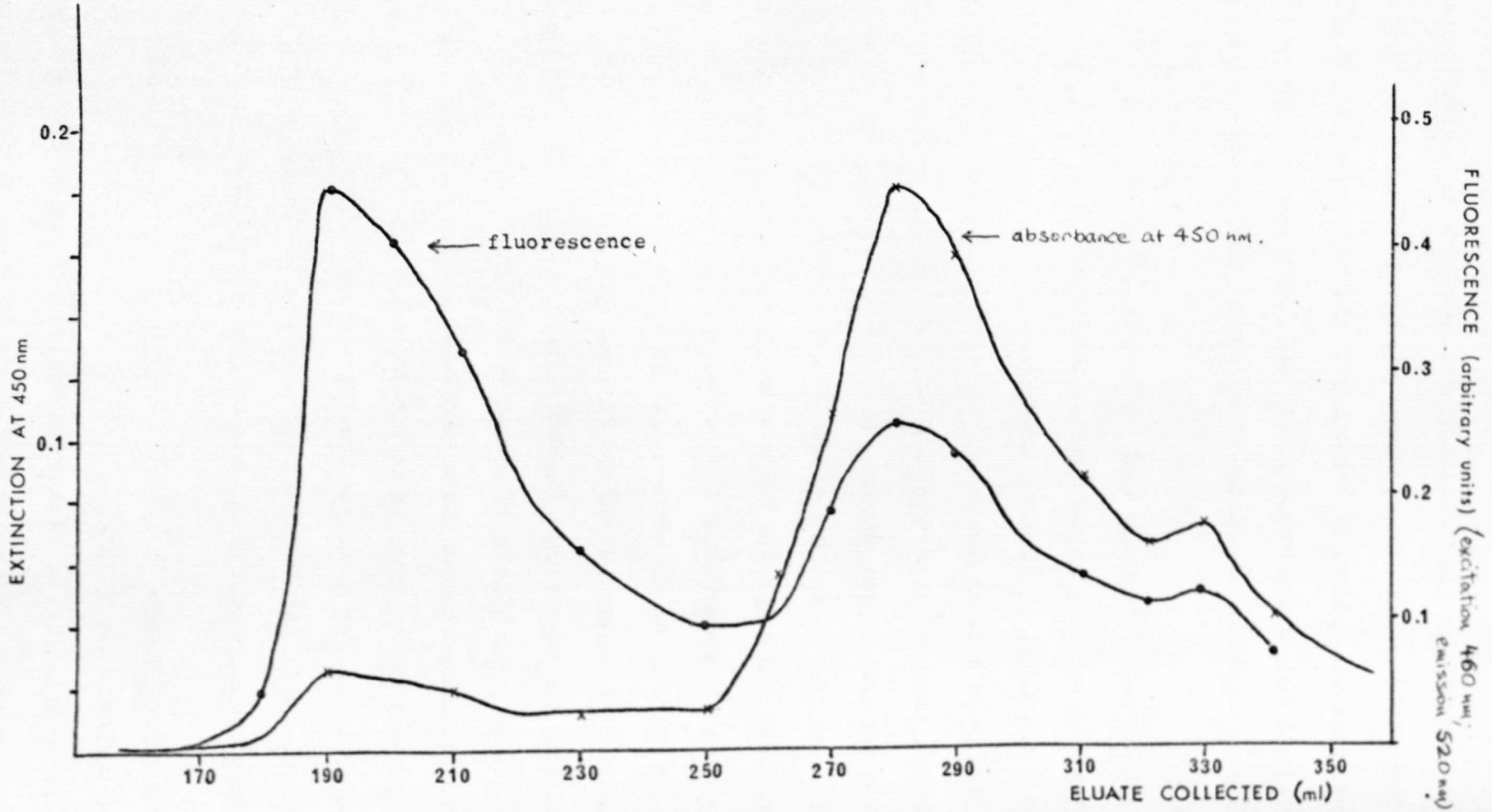
Muller (1941) reported that his enzyme preparation oxidised glucose both aerobically and anaerobically using 2,6-dichlorophenol indophenol (DCPIP) as electron acceptor. Keilin and Hartree (1946) have confirmed this as has Kusai, who found that anaerobic oxidation of glucose occurred at a rate only 3.3% of that in the presence of oxygen. He also showed not only that the FMN content of his FMN-enzyme could be reduced by a third on addition of glucose, but that anaerobic reduction of added dye was faster with the FMN-enzyme than with the FAD-enzyme. Swoboda (1969a) showed that for glucose oxidase from A. niger FMN does not bind to the apoprotein. In experiments where apoenzyme was added to a mixture of FMN and FAD, and the mixture assayed immediately for regenerated glucose oxidase activity, FMN was found not to inhibit the binding of apoenzyme and FAD. Moreover, in sedimentation experiments the sedimentation pattern was the same for apoenzyme and FMN together as it was for apoenzyme alone. The pattern of sedimentation for apoenzyme and FAD together was quite different. Pazur & Kleppe (1964) noted on reactivating apoenzyme with FAD and FMN, that only the incubated FAD plus apoenzyme had glucose oxidase activity.

Methods and materials.

a) The purification of FAD.

The purification of commercial FAD (Sigma) was carried out by the chromatography on a DEAE-cellulose column as described by Massey and Swoboda (1963). However, gradient elution was used with phosphate buffer between 0.1 M and 0.6 M. For all yellow fractions from the

Fig.3.2. The elution pattern of chromatographed commercial FAD.



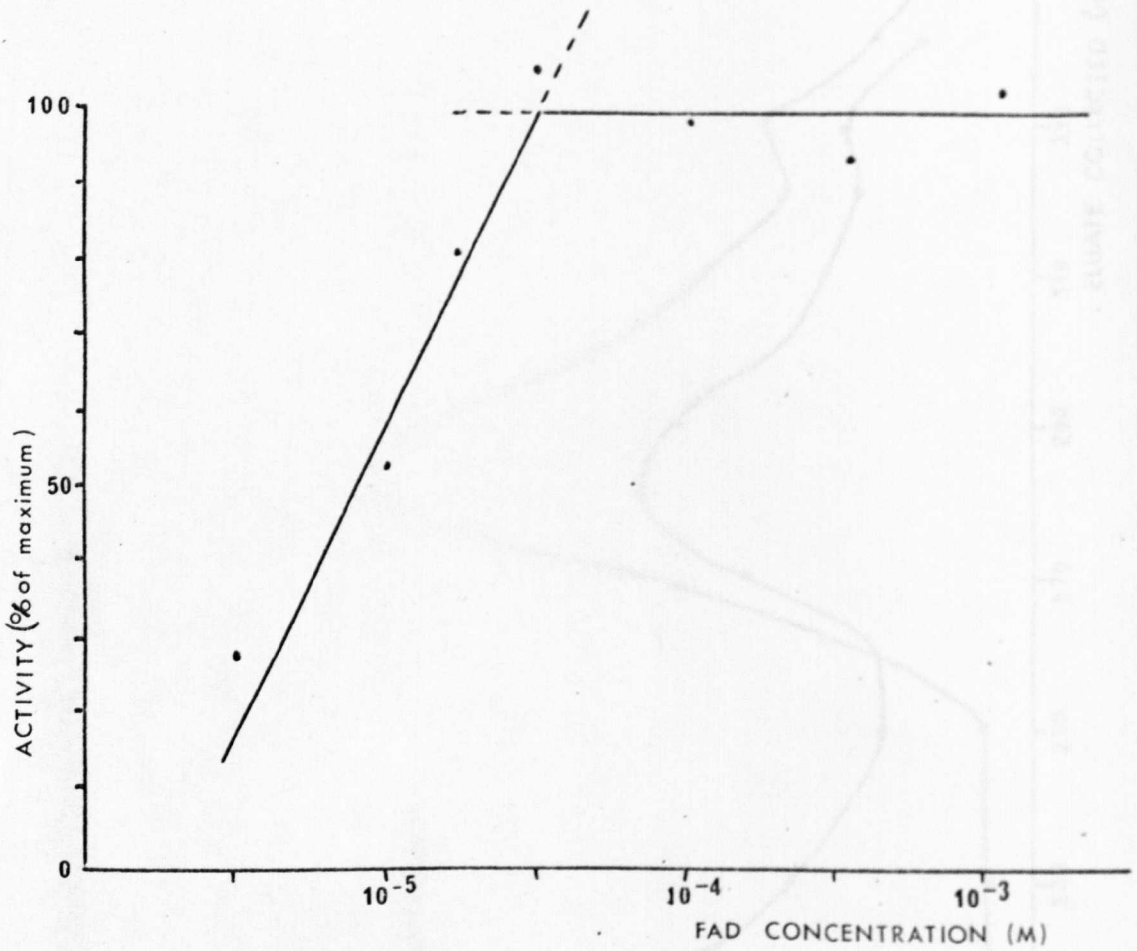


Fig.3.3. Titration of apoenzyme from A.niger glucose oxidase with FAD, apoenzyme concentration was 2.63×10^{-5} M.

column the absorbance at 450 nm was measured. The fluorescence of these same fractions was measured in a Farrand spectrofluorimeter mark II (Farrand Optical Company Inc.) using an excitation wavelength of 460 nm and measuring the emission at 520 nm. The elution pattern is shown in fig.3.2.

b) Preparation of apoenzyme from P. amagasakiense glucose oxidase.

The methods of Kusai (1960) and Swoboda (1969a) were used. Both methods utilise acid-ammonium sulphate treatment under slightly different conditions of temperature and pH. In each case the final precipitate was assayed for residual glucose oxidase activity and for regenerated activity after incubation of the precipitate with 10^{-3} M-FAD for 30 min at 25°. Details of the assay are given in "Methods" chapter 2.

The method of Komai, Massey and Palmer (1969) was tried, but without the initial dialysis, which was designed to remove the contaminating phosphate from their enzyme preparation.

c) Preparation of apoenzyme from A. niger glucose oxidase.

The method of Swoboda (1969a) was used. The final precipitate was assayed as described in (b) above and the results shown in table 3.1. Titration of the apoenzyme with FAD was carried out by incubating equal volumes of 2.5×10^{-5} M-apoenzyme and FAD (various concentrations) for 30 min at 25°C. The mixtures were then assayed for glucose oxidase activity, and a graph was plotted of activity against concentration of FAD (fig.3.3.)

Results.

The results of the chromatography of FAD are shown in fig.3.2. Two peaks of yellow, fluorescent material were eluted from the column. The ratio of fluorescence to absorbance at 450 nm of the first peak was ten times greater than that of the second peak. Hence the first peak was FMN and the second was FAD.

Attempts to prepare the apoenzyme of glucose oxidase from P. amagasakiense proved unsuccessful using both the Kusai and Swoboda

Table 3.1.

Results of an apoenzyme preparation using A. niger glucose oxidase.

	Protein (biuret) mg/ml	Volume ml	Specific activity μ mol/min/mg
Holoenzyme	14.0	4	36.0
Apoenzyme	4.0	3	0
Apoenzyme after incubation with 10^{-3}M-FAD	2.0	6	12.7

Recovery of specific activity = 30%

Yield of apoprotein = 21%

methods. In each case when the final precipitate was tested for activity in the absence of added FAD, activity was found. This activity was not significantly enhanced by incubating the precipitate with excess FAD before assaying for activity. It was noticed that most of the precipitate was still yellow even after five cycles of the Kusai or Swoboda procedures.

An apoenzyme preparation by Dr.Swoboda using the method of Komai and coworkers, yielded about 10% apoprotein. However, the small quantity of holoenzyme available prevented further attempts at apoenzyme preparation. The results of a typical apoenzyme preparation of A.niger enzyme using the Swoboda method are given in table 3.1. It was observed that most of the yellow flavin was removed from the enzyme during the first treatment by acid-ammonium sulphate at -5^{o}. Not more than three cycles of the procedure were required in total to remove the FAD content of the holoenzyme. The results of the titration of the apoenzyme with FAD are shown in fig.3.3. From this it can be seen that the end point of the titration was 5×10^{-5} M-FAD for the 2.5×10^{-5} M-apoenzyme present. This gives a maximum binding of 2 mol FAD/mol apoenzyme.

Discussion

No preparation of P.amagasakiense apoenzyme produced sufficient yield for further experiments. Using A.niger apoenzyme it was confirmed that there are 2 moles FAD per mole protein in the enzyme.

Swoboda (1969a) discussed the possibility of the apoenzyme existing in two forms P_1 and P_2, both of which were able to combine with FAD as follows:-

$$P_1 \rightleftharpoons P_2 \quad \text{.....(i)}$$

$$P_1 + FAD \rightleftharpoons P_1\text{-}FAD \longrightarrow P_2\text{-}FAD \quad \text{.....(ii)}$$

$$P_2 + FAD \longrightarrow P_2\text{-}FAD \quad \text{.....(iii)}$$

Kusai (1960) showed that whereas the P.amagasakiense apoenzyme combined with both FMN and FAD, only the latter reactivated the enzyme

towards glucose and oxygen. However, the newly-synthesised FMN-enzyme was more effective in the DCPIP assay than was the FAD-enzyme.

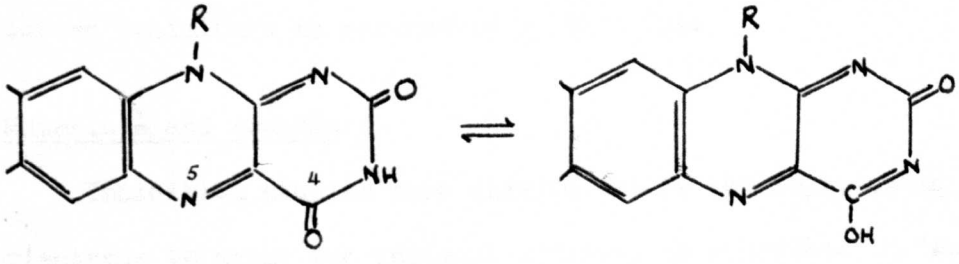
It had been hoped to distinguish between mechanisms (ii), and (i) and (iii) above, by investigating DCPIP and oxidase activity as the apoenzyme combined with FAD and FMN at low temperatures. It has been shown by Kalse and Veeger (1968) and Visser and Veeger (1968) that in the case of lipoamide dehydrogenase there are two stages of regeneration of activity. When the apoenzyme monomer and FAD are incubated together the reconstituted enzyme has high DCPIP activity and low activity towards oxidised lipoate. Prolonged incubation gives holoenzyme (possibly a dimer) with low DCPIP activity but high activity towards oxidised lipoate.

CHAPTER FOUR ENZYME INHIBITION BY HEAVY METALS

Introduction

The sensitivity of proteins to metals at very low concentrations often results in denaturation and accompanying structural changes of the protein molecule. In some cases the effect of these metals in bringing about changes in the secondary and tertiary structure of the proteins has been correlated with the catalytic oxidation of sulphydryl groups in the protein. For example, Casola, Brumby and Massey (1966) and Casola and Massey (1966) have shown that catalytic oxidation by metal ions of sulphydryl groups in the enzyme lipoyl dehydrogenase results in a change in the activity of the enzyme. Inhibition of enzymes by metals may be due to structural changes in the protein chain, either around the active site or at a site distant from the active site but involved in the catalytic activity of the protein, the catalytic site.

In addition to the effects of metals on the protein constituents of an enzyme, there are some reports of the complexing of metals to co-enzymes. Albert (1953) reported a series of studies on the avidity of some natural substances for trace metals. These natural substances included riboflavin, a precursor of FAD, which showed a greater avidity for the ferrous ion than for the cupric ion. In each case the avidity is thought to have been due to the presence of an ionisable hydroxyl group at the C-4 position, peri to the tertiary heterocyclic nitrogen atom at position 5 in the isoalloxazine nucleus of the riboflavin molecule.



Bamberg and Hemmerich (1961) have investigated complexes of silver and isoalloxazine. Their results have shown the nature of the interaction between the ions of silver (1), copper (11) and mercury (11) but with no other metal ions. Degtyar and Gulii (1967) have reported that while glucose oxidase from P. vitale is inhibited by mercuric inhibitors, it is activated by calcium and manganese salts. This latter finding is rather surprising since Komai, Massey and Palmer (1969) have used high concentrations of calcium and of manganese to prepare deflavo xanthine oxidase. They found that these salts caused hydrolysis of FAD.

Kusai (1960) has shown that for P. amagasakiense enzyme the catalytic activity is strongly inhibited by sulphydryl reagents such as pCMB, $HgCl_2$, AgCl, $CuSO_4$. For the A. niger enzyme species Swoboda and Massey (1965) could not detect any sulphydryl groups in either the oxidised or reduced native enzyme when they reacted the enzyme with p-mercuri-acetate and measured the excess mercurial compound polarographically. After denaturation by heat or urea they obtained evidence of one cysteine (SH) and two cystine (S-S) residues per molecule of enzyme. Kleppe (1966) confirmed that this glucose oxidase species was not inhibited in either the oxidised or reduced form by mercurial compounds. Nakamura and Fujiki (1968) have carried out a complete amino-acid analysis of the glucose oxidases from A. niger and P. amagasakiense and have found that there are 7 sulphydryl residues per molecule of enzyme from either source.

Nakamura and Ogura (1968) have obtained inhibition of activity of the A niger species by mercury (II), silver (I) and copper (II) salts, and by pCMB and p-mercuriacetate. They have shown that the effect of these two latter inhibitors is reversible by dilution.

Materials and methods

Inhibition studies were carried out in two ways, using the oxygen electrode to assay for residual activity as described in "Materials and Methods", chapter 2.

(i) Preincubation method.

The enzyme and inhibitor were incubated together in the electrode cell for various lengths of time before the addition of glucose to initiate the reaction.

(ii) Non-incubation method.

With the glucose and inhibitor in the cell, the enzyme solution was added last of all to start the reaction.

The first method was used to obviate the lag period in inhibition experiments. The latter method should give identical results if the inhibition is reversible. If part of the inhibition was a slow reaction, then the preincubated assay would show a different rate of initial activity to that of the non-incubated assay.

In some experiments potassium cyanide (10^{-4} M-final concentration) was added to inhibit any traces of catalase with which the enzyme might be contaminated. Such a contamination would upset the stoichiometry of the reaction by regenerating oxygen in the electrode cell.

Most experiments were carried out in 0.1 M-phosphate buffer pH 6.0 at 25°C. In experiments with pCMB 0.1 M-pyrophosphate buffer pH 7.0 was used. With copper sulphate as inhibitor 0.01 M-acetate buffer pH 6.0 was used. The final enzyme concentration was 3.0×10^{-9} M (active site concentration).

Results

a) Effect of time of incubation on inhibition by $HgCl_2$

The effect of incubating enzyme and inhibitor for various times before assaying for activity is compared with no preincubation in the following table.

The effect of preincubation on inhibition

Preincubation (min)	0	1	5	10	15
% inhibition (10^{-5}M-$HgCl_2$)	17	19	21	17	17

Similar results were obtained at higher inhibitor concentrations. It can be seen that no difference was observed in the percentage inhibition obtained using either the preincubation method or the non-incubation method.

b) Effect of concentration of mercuric salts on the extent of inhibition.

The results are summarised in the following table. Five minutes preincubation was used throughout these assays.

Inhibition by various mercuric salts

Inhibitor final conc.(M)	10^{-4}	10^{-5}	10^{-4+}	10^{-5+}
Inhibitor	Inhibition (%)			
$HgCl_2$	81	19	88	76
$HgBr_2$	23	-	-	-
$Hg(NO_3)_2$ basic	72	45	-	-
pCMB	88	75	96	96

+ values from Kusai 1960

Various concentrations of $Hg(CN)_2$ had no inhibitory effect

c) Effect of KCN on the inhibition of activity

As can be seen from the following table, the presence of a ten-fold excess of cyanide in inhibition assays caused reversal of inhibition

The effect of KCN on inhibition

		% inhibition
10^{-4} M-$HgBr_2$	+ enzyme	23
"	+ 10^{-3} M-KCN + enzyme	0
10^{-3} M-$HgBr_2$	+ enzyme	93
"	+ 10^{-2} M-KCN + enzyme	0

This reversal of inhibition was also demonstrated in the following way. An inhibition assay was set up as described in "Materials and Methods" and the progress of the assay was followed on a pen recorder. After 100 sec the addition of a ten-fold excess of cyanide over inhibitor was made to the electrode cell. A change in the rate of oxygen uptake by the enzyme was seen immediately. This is shown in fig.4.1.

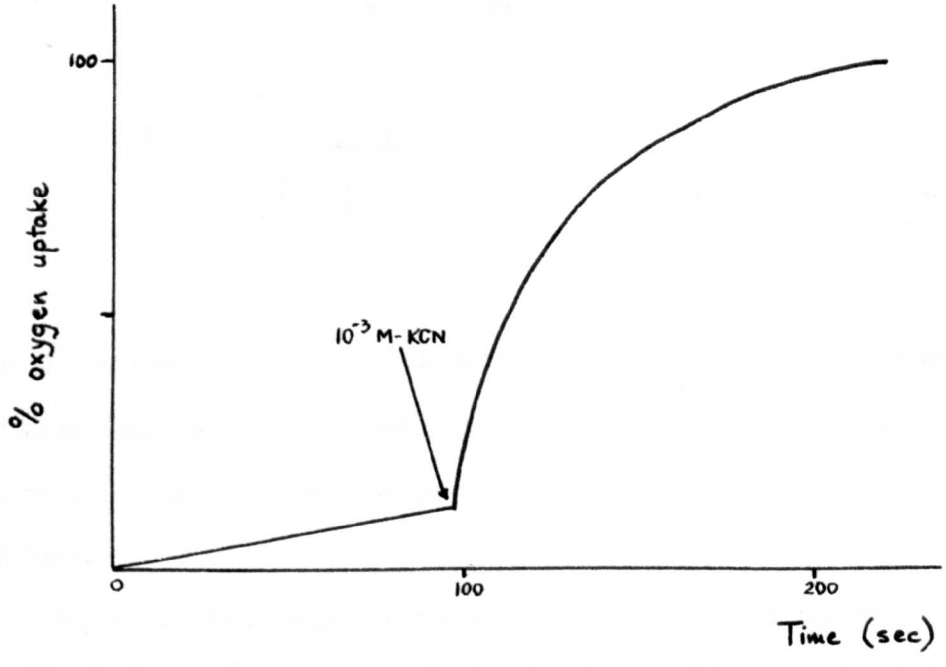


Fig.4.1. Addition of 10^{-3} M-KCN to an inhibition assay containing 10^{-4} M-$HgCl_2$.

In this experiment the inhibition assay showed that the enzyme had lost 88% of its activity. On addition of the ten-fold excess of KCN glucose

oxidase activity was restored to 100% as if no inhibitor was present.

d) Effect of inhibition by silver salts

In all cases when the silver salt was dissolved in 0.1 M-phosphate buffer pH 6.0, a fine yellow precipitate was formed. This may have been silver triphosphate, which is a yellow salt, and it rendered the inhibitor solution unusable. It is likely that silver salts are not precipitated by other buffers but none were tried. Kusai (1960) reported that silver chloride brought about 88% inhibition at 10^{-4}M.

e) To investigate whether cation inhibition is due to a dipole or metal ion effect.

For a metal salt ML consisting of metal M and ligand L, the following equation can be applied to its dissociation:-

$$M^+ + L^- \rightleftharpoons ML$$

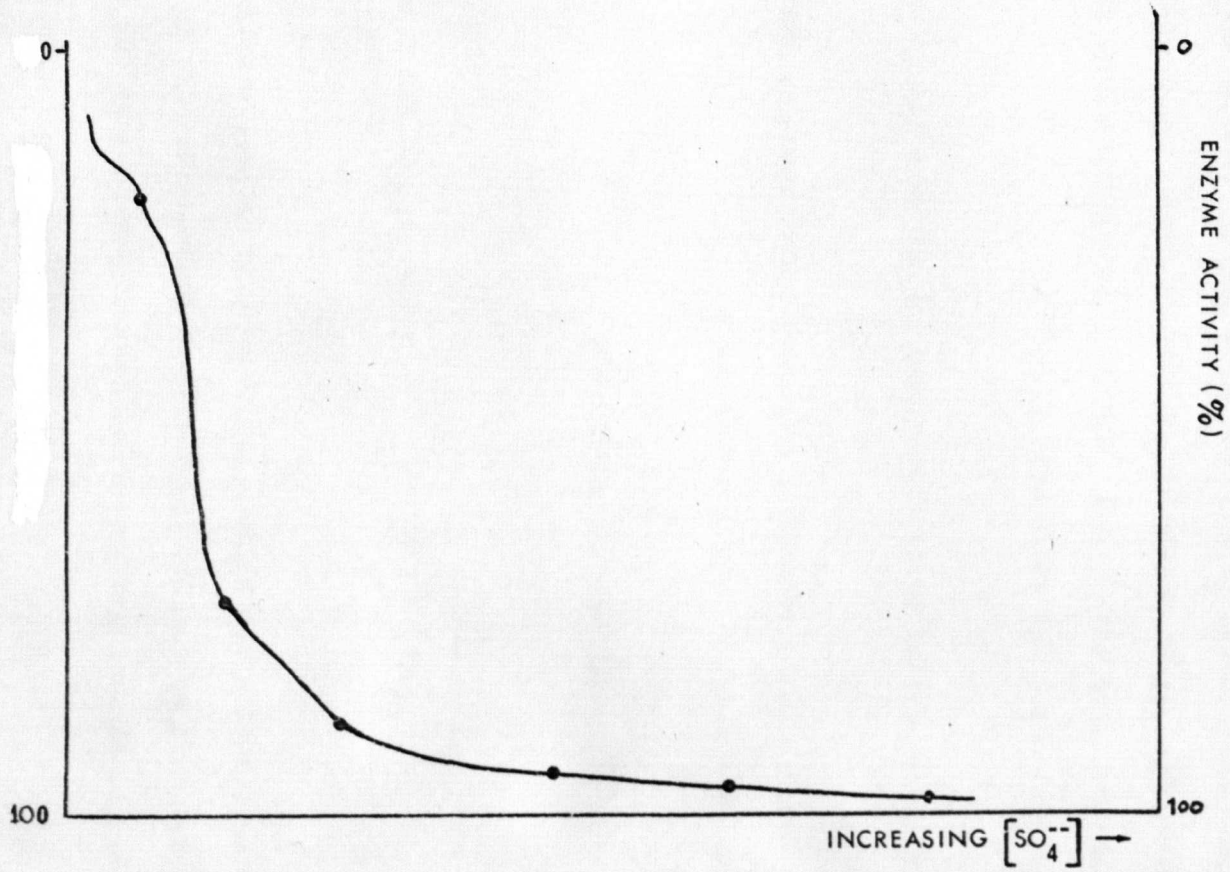
$$K_{eq} = \frac{[ML]}{[M^+]\,[L^-]}$$

where K_{eq} is the equilibrium constant

When the concentration of undissociated salt (ML) is held constant in solution there will be a fixed proportion of dissociated salt in the form of metal ion and ligand, the proportion being determined by the equilibrium constant.

Copper sulphate was chosen as an easy inhibitor to use. Kusai (1960) obtained 30% inhibition at 10^{-4} M and 0% at 10^{-5} M. For copper sulphate K_{eq} = 223.9. Thus using a constant concentration of $CuSO_4$ (5×10^{-5}M) in assays, it was possible to carry out enzyme assays at different concentrations

Fig.4.3. Experimental curve to show the nature of cation inhibition using 5×10^{-5} $CuSO_4$-



Oxygen electrode assay conditions: 0.01 M-acetate buffer pH 6.0, 3×10^{-9} M-enzyme active site concentration, 5×10^{-5} M-$CuSO_4$ at 25°C.

of undissociated salt by adding various calculated amounts of sulphate in the form of sodium sulphate to the assay mixture. When the percentage undissociated $CuSO_4$ is plotted against sulphate ion concentration, the following curves can be predicted for the concentrations of free metal ion and salt in the assay mixture.

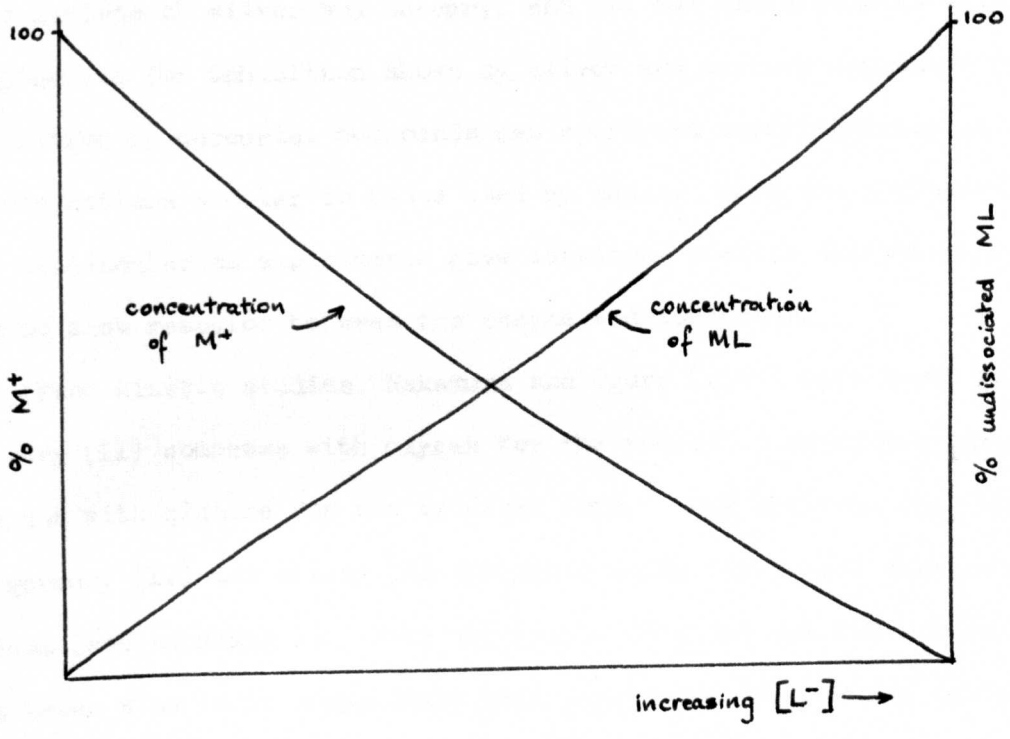


Fig.4.2. Predicted curves for dissociation of metal salt.

As the proportion of ligand is increased in the assay mixture, the amount of free metal ion decreases while the amount of undissociated salt increases. Fig.4.3. shows the experimental curve obtained, and as can be seen, enzyme activity increased as the concentration of free metal ion decreased. That is, the enzyme was inhibited by free metal ions in solution and as the metal ion concentration decreased, the enzyme recovered its catalytic activity. Therefore inhibition by $CuSO_4$ is due to the dissociated copper cation and not to the salt. The presence of weak acetate buffer was thought to have no effect on the copper ion concentration since copper acetate is soluble in aqueous solution up to 0.35 molar.

Discussion

The inhibition of *P. amagasakiense* glucose oxidase by metal salts has been described by Kusai (1960). In this paper he showed that at the same molarity, $CuSO_4$, AgCl and $HgCl_2$ brought about the same degree of inhibition of the enzyme. For copper sulphate it has been shown that inhibition is due to the copper cation. It seems likely therefore that the free cations of silver and mercury, and not the undissociated salts, are responsible for inhibition shown by silver and mercury compounds respectively. Inhibition by mercurial compounds was confirmed experimentally at concentrations similar to those used by Kusai. Both the preincubation and the non-incubation experiments gave identical results indicating that there was no slow reaction between the enzyme and inhibitor.

From kinetic studies, Nakamura and Ogura (1968) have shown that mercury (II) competes with oxygen for the reduced form of the enzyme, and not with glucose for the oxidised form. They analysed the inhibition by mercury (II) and silver (I) compounds using the double reciprocal plot method (see appendix 1). From the graphs obtained the inhibition by silver was shown also to be competitive with oxygen for the reduced enzyme species. This was confirmed by stopped-flow experiments. Using difference absorption spectra they also showed that the change in spectrum of free FAD when mercury (II) was added to it was similar to the difference between the spectrum of the enzyme and that of enzyme + mercury (II) or enzyme + silver (I). However using kinetics to investigate the binding sites of these two metals, they found that the binding sites are different for the metals.

It has been shown that free cupric ions are responsible for the inhibition when $CuSO_4$ is used, and has been suggested that mercuric ions are responsible when mercuric compounds are used as inhibitors.

According to Cotton and Wilkinson (1962) mercuric halides exist almost exclusively (99%) in the undissociated form. Thus although a ten thousand fold gross excess of inhibitor over enzyme was used in these experiments, there would only be very small amounts of free mercuric ion present. The amount present depends on the salt used and its concentration, but using 10^{-5} M-$HgCl_2$ and 3 x 10^{-9} M-enzyme, 10^{-7} M-mercuric ion brought about 81% inhibition. Mercuric bromide dissociates to a smaller extent and so the percentage inhibition was smaller at the same salt molarity.

Cyanide has been used to reactivate mercury inhibited enzymes e.g. yeast enolase (Warburg and Christian 1942). The reversal of mercuric inhibition of glucose oxidase by cyanide may be explained as follows. Mercuric cyanide is very undissociated in water. When cyanide was added to the inhibition assays, the mercuric ions associated with the cyanide ions to form undissociated mercuric cyanide, leaving the enzyme in an uninhibited state.

CHAPTER FIVE. THE EFFECT OF BISULPHITE ON THE SPECTRAL AND FUNCTIONAL PROPERTIES OF P. AMAGASAKIENSE GLUCOSE OXIDASE

Introduction

Swoboda and Massey (1966) have studied the effects of bisulphite addition to A. niger glucose oxidase in terms of its effects on the absorbance and fluorescence spectra of the enzyme. As a result they have postulated the existence of an enzyme-bisulphite complex, which they showed to be inactive with molecular oxygen. The complex reacted very slowly with glucose. The new species was characterised by the bleaching of the visible spectrum of the oxidised enzyme, and the appearance of a new shoulder of absorption at about 330 nm. This new absorption spectrum was similar to but not identical with that of the fully reduced enzyme, indicating that bisulphite was not a simple reducing agent of the enzyme. An equilibrium constant could be calculated for the reaction; the extent of reaction of oxidised enzyme with bisulphite being dependent on the concentration of bisulphite, of enzyme, on the temperature and on the pH of the reaction.

Massey and Muller (1969) and Massey et al (1969) have studied the reactivity of flavoproteins with bisulphite at pH 7.0 and above, when the bisulphite has dissociated to sulphite.

$$H_2SO_3 \overset{K_1}{\rightleftharpoons} H^+ + HSO_3^- \overset{K_2}{\rightleftharpoons} H^+ + SO_3^{--}$$

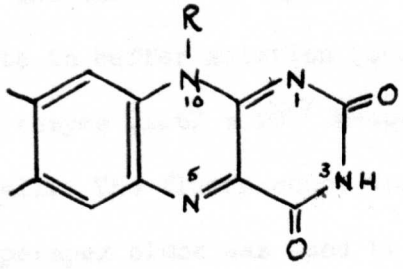
$K_1 = 1.54 \times 10^{-2} M$; $pK_1 = 1.81$

$K_2 = 1.02 \times 10^{-7} M$; $pK_2 = 6.91$

at 18°C

They found that only flavoproteins which reacted catalytically with molecular oxygen as H acceptor could form sulphite adducts. One sulphite molecule was bound per flavin molecule. As a result of studies on model compounds they concluded that the sulphite molecule bound to the N-5 position in the isoalloxazine ring. This confirmed the findings of Kaplan (1960) who worked on addition compounds of sulphite and pyridine

nucleotides, that the sulphite was bound at the N-5 position.



The numbering of the isoalloxazine ring

Materials and Methods

All absorption spectra were measured on a Cary model-14 spectrophotometer, fitted with a sensitive slidewire (0 - 0.1 OD units). Quartz cells were used, the temperature of the cell and contents being maintained at 25°C. Spectra were run from 550nm to 300nm against a water blank, to which bisulphite additions were made when bisulphite was added to the experimental solution. The enzyme was made up in 0.1 M-acetate buffer at the appropriate pH, with 10^{-4} M-EDTA present to remove any traces of metal ions. Each spectrum was recorded 5 min after each bisulphite addition. Spectral measurements were corrected for dilution of the sample by successive bisulphite additions. Prior to each set of experiments, the enzyme solution was spun at 50,000g in a Beckman L-2 centrifuge for 60 min.

Any denatured protein and dust particles were precipitated as a sediment in the centrifuge tube, and only the supernatant was used in spectrophotometric experiments in order to overcome opalescence problems.

Enzyme activity studies were performed using an oxygen electrode (see "Materials and Methods", chapter 2.) 0.1 M-acetate buffer containing 10^{-4} M-EDTA was used.

Fluorimeter experiments were performed in a Farrand spectrofluorimeter Mark II (Farrand Optical Co.Inc.), using an excitation wavelength of

295 nm, and measuring the emission at 350 nm. Slit widths of 5 mm were used for the excitation light path and 20 mm for the emission path. 1 ml bisulphite in buffer solution (containing 3×10^{-3} M-EDTA) was added to 2 ml enzyme (1.67×10^{-7} M-active site concentration) in a fluorimeter cell. The fluorescence was measured before and after addition. A perspex block was used to check and adjust the fluorescent emission of the lamp to an arbitrarily fixed standard. The temperature of the cell and contents was maintained at 25°C.

Fresh bisulphite solutions were prepared daily from 5 ml ampoules of 35% sodium bisulphite solution (British Drug Houses Ltd., micro-analytical grade) to overcome the problem of slow air oxidation of bisulphite.

Results

Two control experiments were performed on bisulphite solutions, to test the stability of the solutions towards oxidation by air in solution, and towards cyanide in oxygen electrode assays. The results of these control experiments were utilised in subsequent experiments using bisulphite solutions.

Metal ions can be shown to catalyse the oxidation of bisulphite, by performing an experiment as in a) below with just bisulphite and heavy metal ions in solution in the electrode cell. Therefore in all subsequent experiments with bisulphite no cyanide was added, and unless otherwise stated, EDTA was present in a ratio of 1:50 to bisulphite concentration.

a) Control experiment on the oxidation of bisulphite solutions.

Bisulphite solutions were injected into the cell of an oxygen electrode containing buffer (0.1 M-acetate pH3-5 or 0.1 M-phosphate pH 6.0). The uptake of oxygen, corresponding to the oxidation of bisulphite by dissolved

oxygen was followed on a moving-pen recorder. EDTA prevented this oxidation of bisulphite when present at a concentration of not less than 1 mole EDTA per 50 moles bisulphite (Ford-Hutchinson 1969) during the period of the experiment (5 min). The EDTA probably functioned by removing traces of metal ions from solution, since some metal ions catalyse bisulphite oxidation.

b) Control experiment on the effect of cyanide in inhibition assays containing bisulphite.

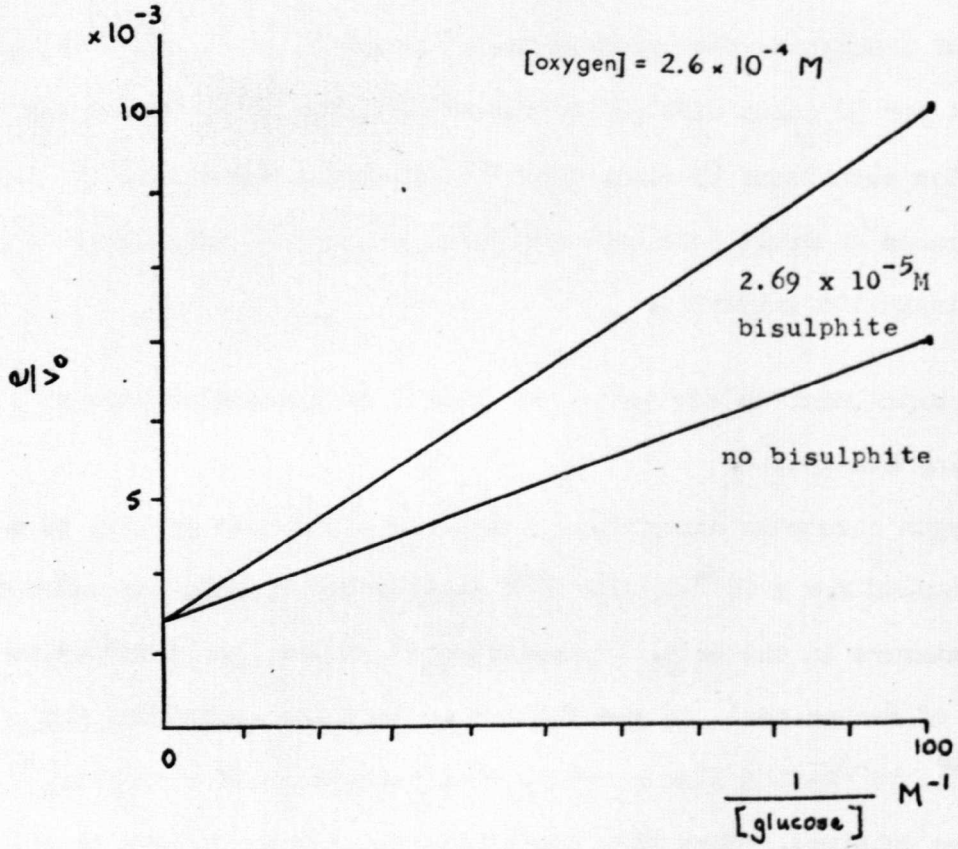
An oxygen electrode assay was set up with bisulphite present at a concentration of 2.0×10^{-4} M, and 10^{-3} M-potassium cyanide was added to the assay mixture in the cell. On addition of enzyme (3.0×10^{-6}M) no inhibition of enzyme activity was observed. When the experiment was repeated with 10^{-3}M-EDTA also present, some inhibition of catalytic activity was obtained. When only bisulphite and excess cyanide were present in the electrode cell, uptake of oxygen was observed. This uptake of oxygen was prevented by addition of EDTA to the electrode cell, to a final concentration of at least 10^{-3} M. The cyanide had been added to inhibit any traces of catalase in the glucose oxidase preparation, which might interfere with the stoichiometry of the reaction. It was of ANALAR reagent grade, but the experiment indicates that minute traces of metal ions must have been introduced into the reaction mixture by the addition of cyanide solution.

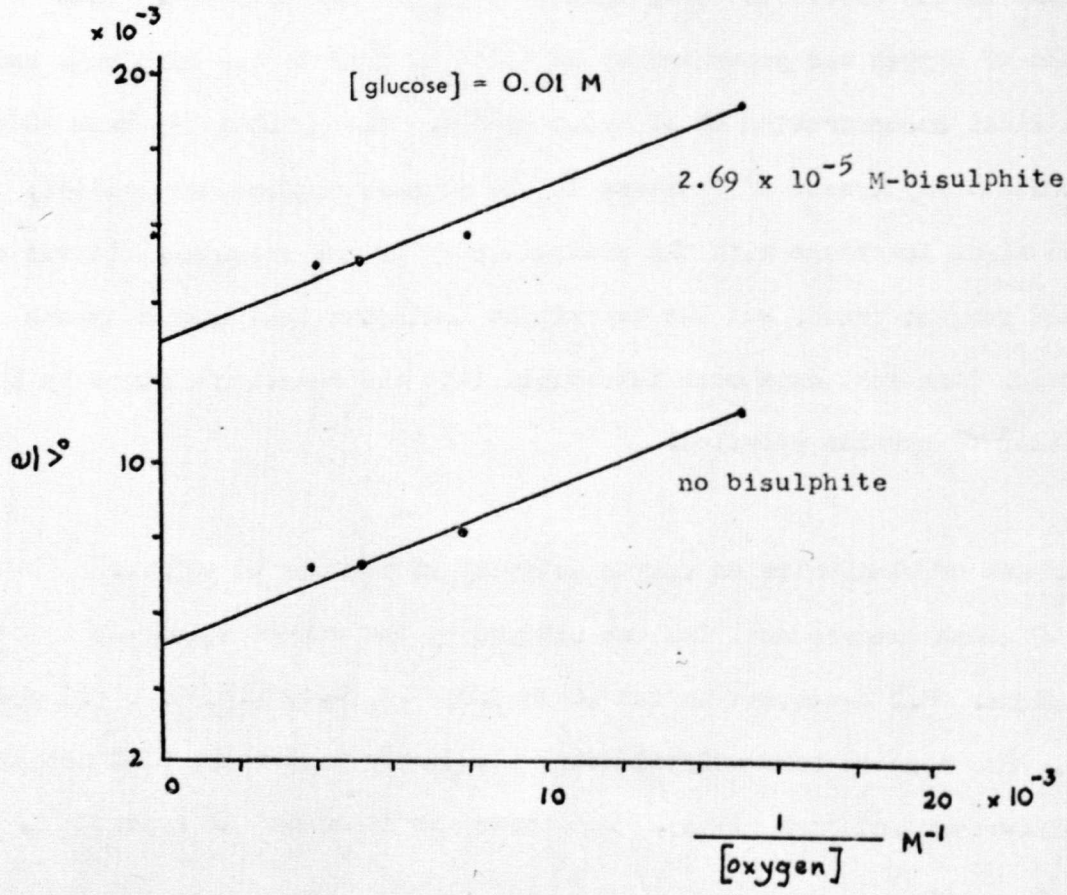
c) Effect of bisulphite on enzyme activity at various pH values.

Glucose oxidase activity was assayed by the oxygen electrode technique. 0.1 M-acetate buffer at pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 was used. The results were analysed using the double reciprocal plot method of Lineweaver and Burk (1934). The theory is described in appendix 1.

Fig.5.1. Reciprocal plots of enzyme activity at pH 3.5.

0.1 M-acetate buffer pH 3.5 , 3.3×10^{-6} M enzyme





A typical set of plots is reproduced in fig.5.1. The values of K_I, the inhibition constant, at various pH values is shown in table 5.1.

Table 5.1. Variation of K_I with pH (oxygen electrode assay).

pH	K_I (M)
5.5	1.97×10^{-4}
5.0	4.03×10^{-5}
4.5	2.09×10^{-5}
4.0	1.12×10^{-5}
3.5	0.89×10^{-5}
3.0	0.95×10^{-5}

(Enzyme concentration = 3.3×10^{-6}M)

The K values were plotted as $-\log_{10} K$ against pH, to give a Dixon plot (Dixon 1953) shown in fig.5.2.

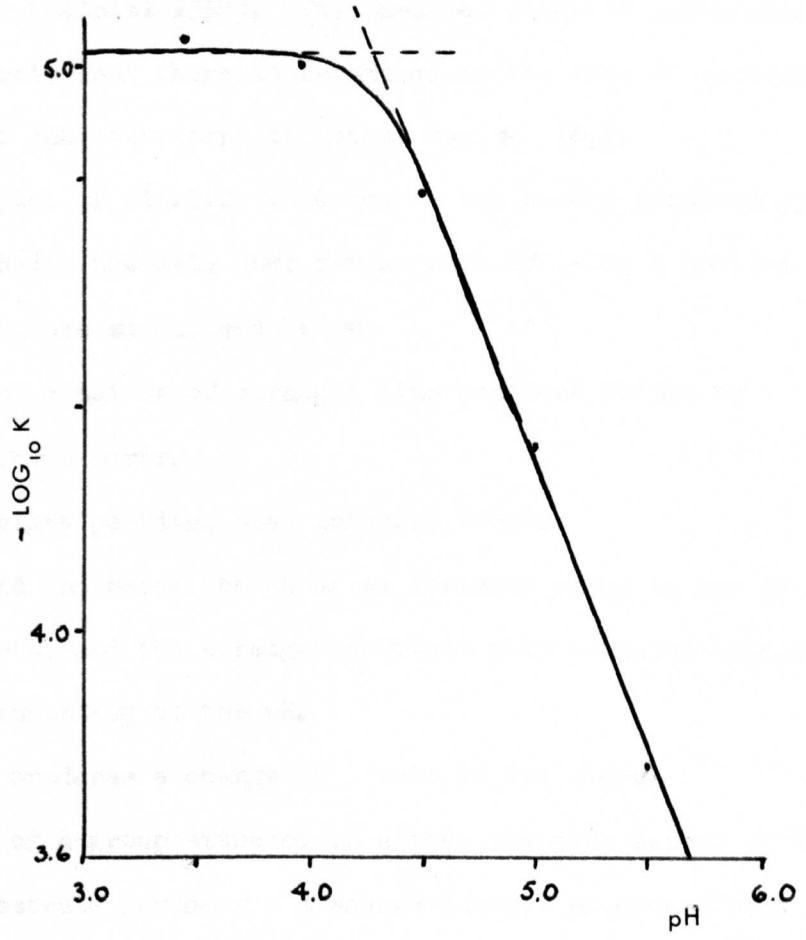


Fig.5.2. Dixon plot of K values obtained from oxygen electrode experiments with bisulphite.

In fig.5.1 for the plot of reciprocal velocity against reciprocal glucose concentration, the resulting lines (for experiments with and without bisulphite present) intercepted on the vertical axis. This indicated that the inhibitor was competing with glucose for the oxidised form of the enzyme. The plot of reciprocal velocity against reciprocal oxygen concentration showed the comparable lines parallel to each other, indicating uncompetitive inhibition (see appendix 1). The simple explanation is that the bisulphite only combines with the oxidised enzyme species, the rate equation for which is

$$\frac{e}{v_o} = \phi_o + \frac{\phi_1}{[G]}\left(1 + \frac{I}{K_I}\right) + \frac{\phi_2}{[O_2]}$$

where e is enzyme concentration, v_o is initial velocity of the reaction, I is concentration of inhibitor, ϕ_o, ϕ_1, and ϕ_2 are rate constants in Dalziel notation (Dalziel 1957). The parallel lines of uncompetitive inhibition indicate that there is no change in the rate of reaction and therefore no inhibitor term is needed for $\phi_2 / [O_2]$.

The Dixon plot of fig.5.2. is based on the theory proposed by Dixon (1953). Rules are laid down for a graph of -log K plotted against pH, which are summarised below

a) The graph consists of straight line sections joined by short curved parts.

b) The straight portions have integral slopes.

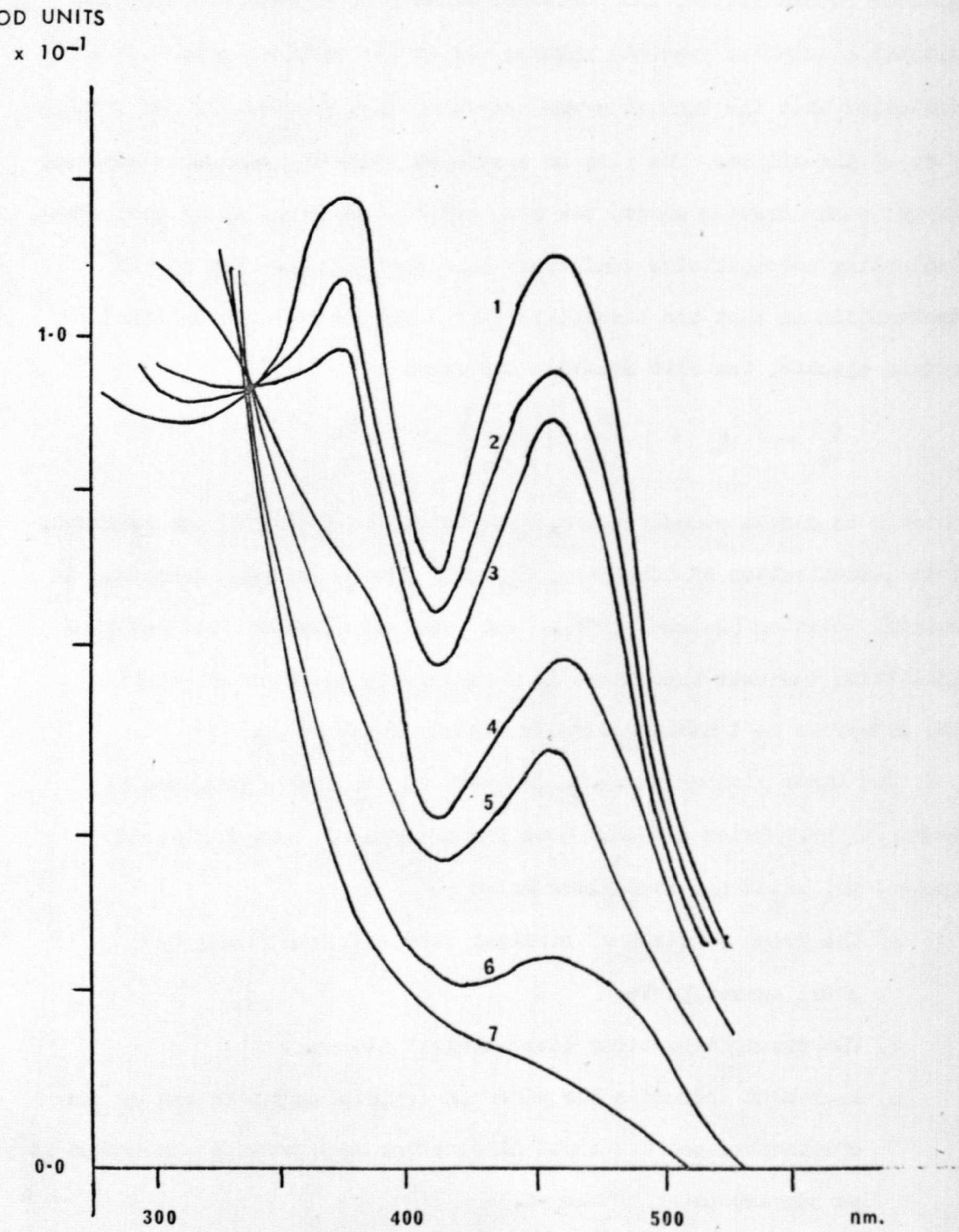
c) Each bend indicates the pK of an ionising group in one of the components, and the straight portions when produced intersect at a pH corresponding to the pK.

d) Each pK produces a change of 1 unit in the slope.

e) Each pK of a group situated in either the free enzyme or the free substrate produces a downward curve; an upward bend indicates a group in the enzyme-substrate complex.

f) The curvature at the bends is such that the graph misses the intersection point of the neighbouring straight parts by a

Fig.5.3. Effect of bisulphite on absorption spectrum.



1. No bisulphite ; 0·1 M-acetate buffer pH 5.0, 0.96×10^{-5} M-enzyme
2. 1.063×10^{-6} bisulphite
3. 7.211×10^{-6} "
4. 2.253×10^{-5} "
5. 3.771×10^{-5} "
6. 9.708×10^{-5} "
7. 1.436×10^{-1} "

vertical distance of 0.3 units.

g) The slope of any straight line section is numerically equal to the change of charge occurring in that pH range when the complex dissociates into free enzyme and free substrate.

From these rules it will be seen that for the graph in fig.5.2. the change in slope = -1, that the graph bends downwards and that the produced straight portions intersect at pH 4.25. The pK for bisulphite dissociating to sulphite is 6.9. Therefore the enzyme contains a singly charged group of pK 4.25, which combines with bisulphite.

d) Effect of bisulphite on the absorption spectrum at various pH.

Successive additions of bisulphite were made aerobically to a solution of enzyme (active site concentration 10^{-5}M) in 0.1 M-acetate buffer at pH 3.0, 3.5, 4.0, 4.5, 5.0. The effect of bisulphite on the absorption spectrum at one pH is shown in fig.5.3. Results similar to those published for A. niger glucose oxidase (Swoboda and Massey 1966) were obtained, and included the observation that the bleached enzyme did not react with oxygen and that molar excesses of bisulphite were required for bleaching.

Since an isosbestic point was obtained, it was assumed that two species were present i.e. enzyme and enzyme-bisulphite complex. From the changes in optical density at a particular wavelength (450nm) the equilibrium concentrations of enzyme, bisulphite and enzyme-bisulphite complex were determined. The dissociation constant, K, was calculated at different pH values

$$K = \frac{[\text{enzyme}]\ [\text{bisulphite}]}{[\text{enzyme-bisulphite complex}]}$$

The theory of the determination of K from spectral data is given in appendix 2.

The K values obtained for a range of bisulphite concentrations at

Table 5.2. Variation of K with bisulphite concentration.

(Enzyme concentration = 4.31×10^{-6}M : pH 5.0)

Bisulphite concn. (M)	% change in absorbance at 450nm	Dissociation constant K (M)
1.063×10^{-6}	8	1.395×10^{-5}
7.211×10^{-6}	15	4.056×10^{-5}
2.253×10^{-5}	43	3.031×10^{-5}
3.771×10^{-5}	54	3.242×10^{-5}
9.708×10^{-5}	77	3.030×10^{-5}

Table 5.3. Variation of K with pH

(Enzyme concn. = 4.88×10^{-6} M)

pH	Dissociation constant K (M)
5.5	1.072×10^{-4}
5.0	3.30×10^{-5}
4.5	2.67×10^{-5}
4.0	7.00×10^{-6}
3.5	5.00×10^{-6}
3.0	7.24×10^{-6}

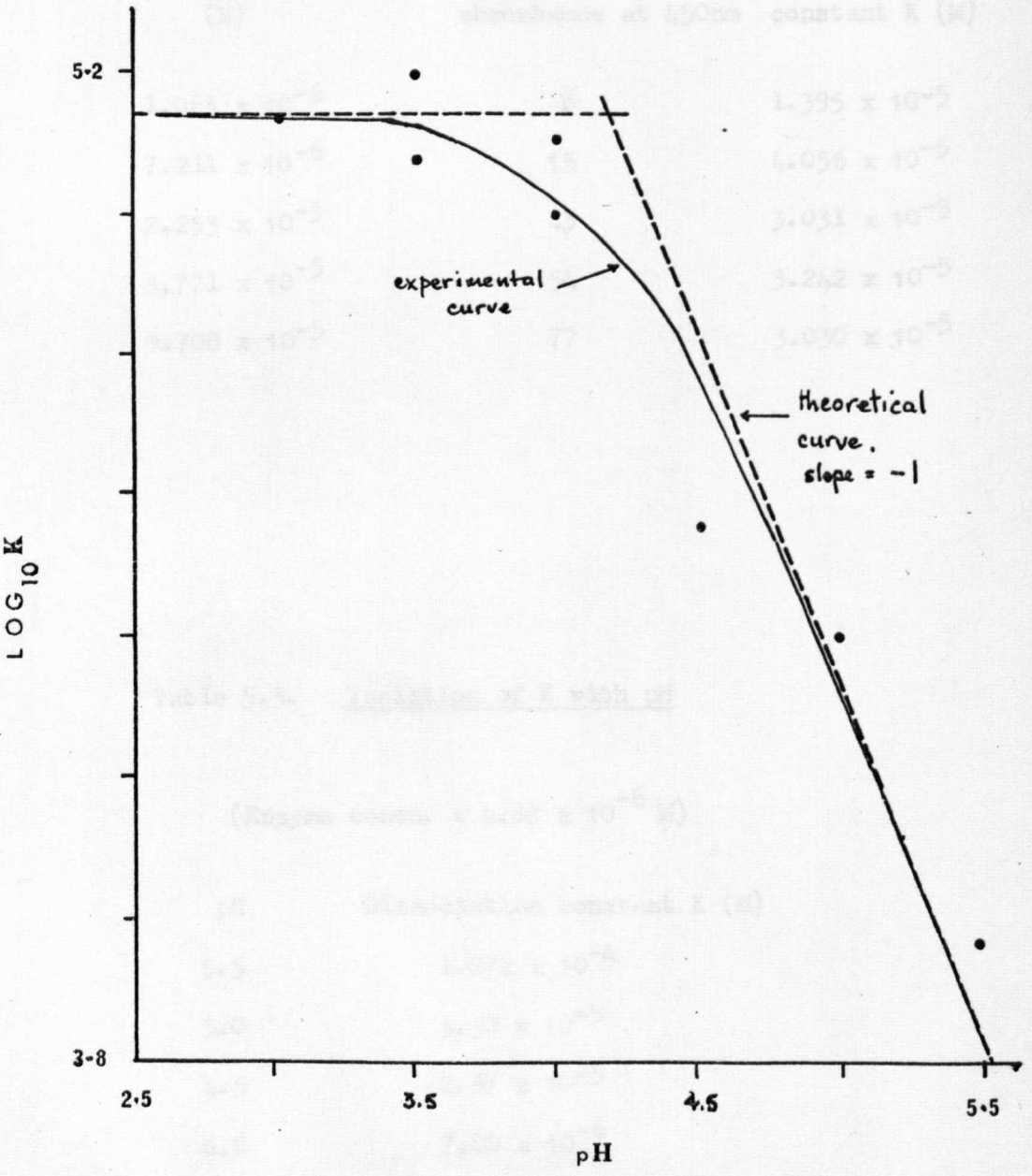
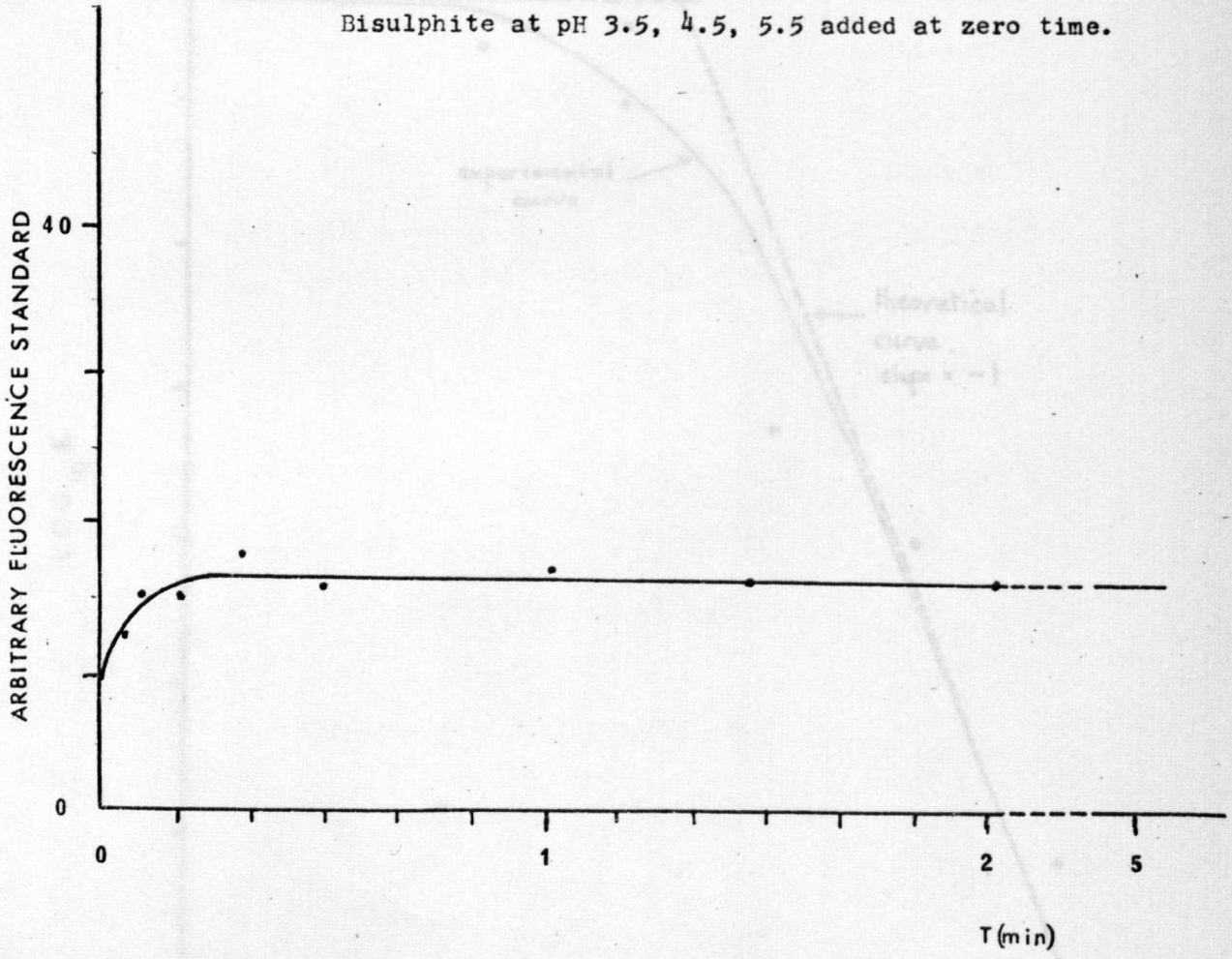


Fig.5.4. Dixon plot of K values obtained from spectra with bisulphite.

Fig,5.5. Variation in protein fluorescence of the enzyme on addition of bisulphite with pH.



1 ml 0.3 M-bisulphite in 0.1 M-acetate buffer added to 2 ml enzyme solution (1.67×10^{-7} M-active site concentration). The bisulphite solution contained 3×10^{-3} M-EDTA. The excitation wavelength was 295 nm, and the emission wavelength was 350 nm.

constant pH are given in table 5.2. It will be seen that K is constant over a range of bisulphite concentrations. The K values obtained at different pH values for constant bisulphite concentration are in table 5.3. These values were plotted as $-\log_{10} K$ against pH, as described in the preceding section, in fig.5.4. The enzyme species is shown to have a singly charged group of pK 4.2.

e) Investigation by fluorescence of the addition of bisulphite to the enzyme at various pH values.

Fluorescence changes at 350 nm, using an excitation wavelength of 295 nm, were measured before and after additions of bisulphite. It is seen in fig.5.5. that the fluorescence of the enzyme doubled on addition of bisulphite in 0.1 M-acetate buffer pH 3.5, 4.5, 5.5. Within experimental error the change in fluorescence was the same at each of the pH values. No change in fluorescence was observed over a period of 2 min. The buffers alone did not bring about a change in protein fluorescence.

f) Investigation of the order of bisulphite addition by fluorescence measurements.

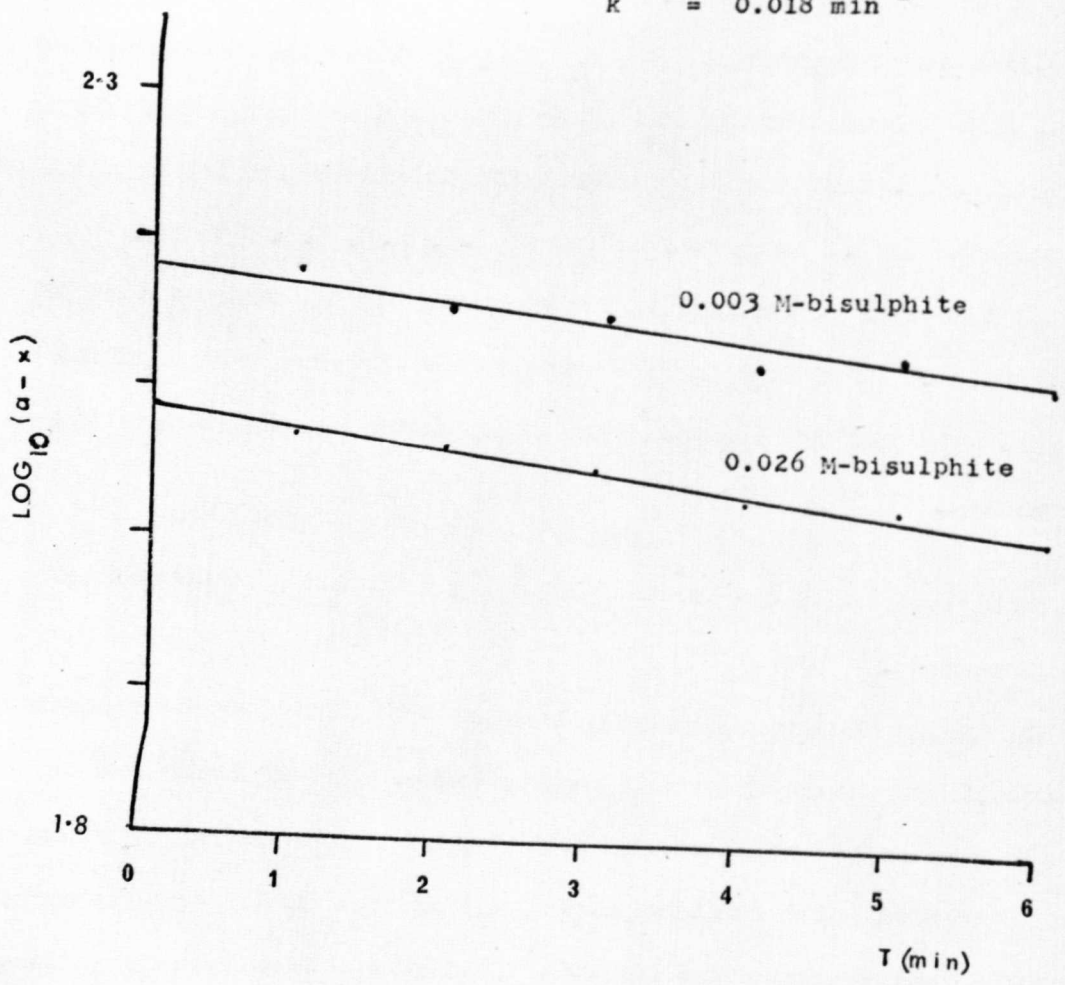
The fluorescence of a protein is extremely sensitive to changes in the protein structure or external environment. The concentration of protein required is smaller than that required for spectral experiments, so that if bisulphite addition caused any changes in the protein structure, or of the environment near the site of addition, it should be reflected in a change in fluorescence.

At pH 3.5 the above fluorescence experiment was repeated over a longer period. An initial rapid rise in fluorescence was obtained, followed by a slow decrease in fluorescence over a period of 5 min, eventually reaching the initial value. Using the equation for 1st order reactions, the slow reaction was analysed.

Fig.5.6. First order plot of bisulphite addition.

$$\log_{10}(a-x) = \frac{-kt}{2.303} + \log_{10} a$$

$$k = 0.018 \text{ min}^{-1}$$



$$\log_e \frac{a}{a-x} = kt$$

where a is initial concentration of enzyme, (a-x) is the concentration of enzyme remaining at time t when the concentration of product is x, k is the velocity constant. The results are shown in fig.5.6. From the shape of the graph it can be seen that the slow reaction follows 1st order kinetics. The velocity constant has a value of 0.018 min^{-1} and is independent of bisulphite concentration.

Discussion

It was found that the oxidation of bisulphite by molecular oxygen in solution was prevented in the presence of EDTA. The addition of cyanide (as potassium cyanide in solution) to the bisulphite appeared to encourage oxidation of the bisulphite. Very small concentrations of metal ions (of the order of 10^{-6}M) will assist this oxidation (Perfetto 1969 personal communication) and it seems likely therefore that the role of EDTA is chelating these metal ions present in the buffer and other air-saturated solutions. The addition of potassium cyanide at concentrations of the same order as that of EDTA, upset the equilibrium and left sufficient metal ions in solution (from trace impurities in the cyanide) to catalyse the oxidation of bisulphite.

The action of bisulphite in bleaching this enzyme was the same as that described by Swoboda and Massey (1966) for the A.niger species. The extent of formation of enzyme-bisulphite complex as proposed by them, was shown to be dependent on pH and on bisulphite concentration for the P.amagasakiense species too. Concomitant with the bleaching of the enzyme was a loss of glucose oxidase activity. This change was irreversible by oxygen and therefore not a reduction reaction. The prosence in the spectra of fig.5.3. of an isosbestic point confirmed the suggestion that an enzyme-bisulphite complex was being formed from 2 different species.

The two-fold increase in fluorescence of the enzyme protein on bisulphite addition may indicate some change in the conformation of the

enzyme. The fluorescence measured at 295/350 nm was that of tryptophan residues in the protein, with a possible small contribution from tyrosine residues. Swoboda (1969a) showed that such an increase in fluorescence could be brought about by the breaking of a weak bond between the enzyme protein and the FAD moiety. However addition of glucose to glucose oxidase also brings about increased fluorescence because of an internal transfer of energy in the enzyme. This allows full expression of the fluorescence which was previously quenched in part.

From the experiment to find the rate constant and order of reaction of bisulphite binding, it appeared that the binding occurred in two stages which could be represented by the following equation:-

$$E + \text{bisulphite (B)} \rightleftharpoons E'B' \xrightarrow{k} EB$$

Bisulphite was at a concentration a thousandfold greater than the dissociation constant (K) value. Hence the first reaction (of increased fluorescence) was very fast, while the second was a slow reaction obeying 1st order kinetics, and having a velocity constant $k = 0.0003\ sec^{-1}$. It is possible that metal ions, present as a contaminant, were catalysing the oxidation of bisulphite in the reaction solution and thus reducing the amount of complex formed (if any). This theory is supported by the fact that the slow decay of fluorescence continued until the initial value of that for the enzyme alone was reached.

The dissociation constant was found to be dependent on pH (table 5.3.) as shown by Swoboda and Massey(1966) for A.niger. The effect of hydrogen ion concentration on enzyme-bisulphite complex formation can be represented by the equation

$$E_o + H^+ \rightleftharpoons E_oH^+ + HSO_3^- \rightleftharpoons E_oH^+(HSO_3)^-$$

This mechanism has been proposed for the P.notatum enzyme (Bright and Appleby 1969) and for A.niger enzyme (Weibel and Bright 1971). E_o represents the oxidised enzyme species which at low pH becomes protonated and susceptible to binding of anions. Glucose does not bind to the

protonated form. The dissociation constant for the complex

$$K = \frac{[\text{enzyme}]\ [\text{bisulphite}]\ [H^+]}{[\text{enzyme-}H^+\text{- bisulphite complex}]}$$

when the K values were plotted as $- \log_{10} K$ versus pH in a Dixon plot, two straight intersecting lines were obtained using the Dixon rules (Dixon 1949, 1953) one horizontal and the other with a slope of -1. The intersection corresponded to pH 4.2 (fig.5.2 and 5.4.) which according to the Dixon rules applied to the plots represented the pK of an ionising group in the enzyme (bisulphite has a pK of 6.9).

Only two possible ionisations are available

(i) the carboxyl group of an acidic amino-acid

$$-COO^- + H^+ \rightleftharpoons -COOH$$

The β-carboxyl of aspartic acid has a pK of 3.86, while that of glutamic acid is 4.25. However the increased inhibition of anion with increasing hydrogen ion concentration excludes this possibility because increasing H^+ (in the above equation) would cause less carboxyl groups to remain ionised. Also it would not be expected that anions eg bisulphite would combine with negatively charged carboxyl residues but rather be repelled by them.

(ii) ionisation of adenine of the FAD moiety

$$-NH_2 + H^+ \rightleftharpoons -NH_3^+$$

This ionisation of the adenine amine group has a pK of 4.1 (Bendich 1955). This seems a more likely ionisation to take place in the enzyme, in view of the anionic nature of the inhibitor which would be attracted to the charged amine group. Also the adenine is sterically close to the N-5 position of the iso alloxazine ring, near which glucose oxidation is thought to occur (Brown and Hamilton 1970, Weibel and Bright 1971)

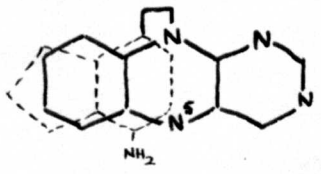
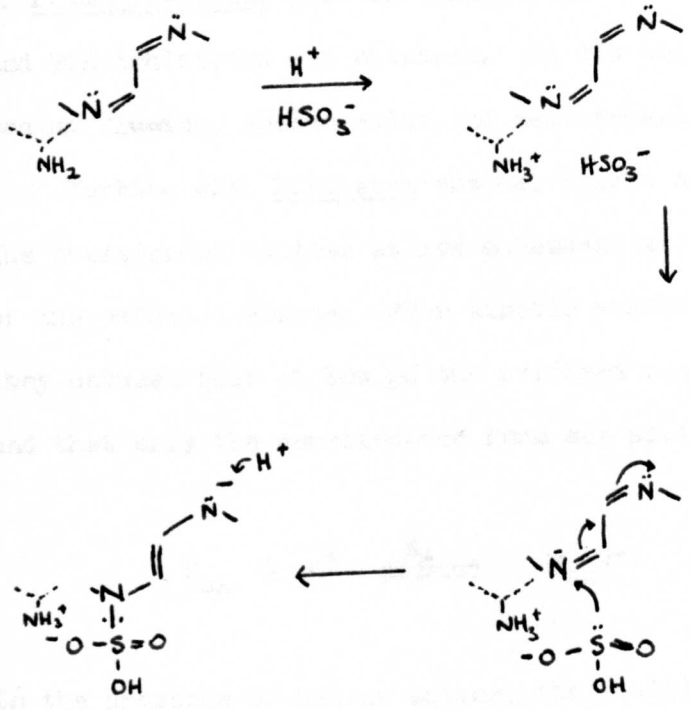


Diagram of the planes of the isoalloxazine ring and adenine of FAD

In a position adjacent to, or in association with the adenine amino group, the bisulphite might block the attachment and subsequent oxidation of glucose. Hence bisulphite competes with the glucose, as confirmed by the Lineweaver-Burk plots.

Since bisulphite brings about a bleaching of the enzyme, its position must be sufficiently close to the N-5 of the isoalloxazine ring to donate its electron pair even to the extent of forming a bond. A suggested mechanism is



The reaction of the reduced enzyme with oxygen is prevented because of the irreversible blocking of the N-5 position by the bisulphite molecule. It is noted that in the above mechanism the character of the $-\overset{5}{N}-C=C-\overset{10}{N}-$ of the isoalloxazine ring is the same as that of reduced FAD.

Introduction

It was observed that at low pH, chloride ions inhibited *P. amagasakiense* glucose oxidase activity. A further investigation was carried out at low pH into the effect of halide and other anions on the enzyme. Results for the bisulphite ion are included in chapter 5.

Kusai (1960) has shown that at pH 5.6 hydroxylamine, hydrazine, phenylhydrazine, sodium bisulphite and dimedone are all good inhibitors of *P. amagasakiense* glucose oxidase. At 10^{-2} M concentrations between 60 and 90% inhibition was obtained. He did not obtain inhibition with sodium fluoride, sodium azide and semicarbazide.

Working with *P. notatum* enzyme, Bright and Appleby (1969) found that the presence of halides at low pH caused an increase in the pK_I of the oxidised enzyme. From kinetic studies in the absence of halide, they deduced that at low pH the oxidised enzyme species became protonated and that only the unprotonated form was active.

$$E_{ox} + H^+ \underset{}{\overset{K_I}{\rightleftharpoons}} E_{ox}H^+ \qquad (i)$$

In the presence of halide anions, the equilibrium represented by equation(i) was shifted to the right because the protonated form of the enzyme preferentially combined with the anions. A change in the pK_I from 4.0 to 5.0 was obtained.

Equation (i) was confirmed for *A. niger* enzyme by Weibel and Bright (1971) and it was shown that the enzyme species $E_{ox}H^+$ had little or no affinity for glucose. The effectiveness of halides in bringing about a change in the maximum turnover number (k_{cat}) was found to be $F^- \gg Cl^- \approx Br^-$.

Materials and Methods

All experiments which were carried out using an oxygen electrode were as described in chapter 2. In inhibitor assays the inhibitor and enzyme were preincubated for 5 min before addition of glucose to initiate the reaction. EDTA (10^{-3}M-final concentration) was present in all assays to remove any metal ion impurities which might interfere with the assay. 0.1 M-acetate buffer was used at pH 5.5 and 3.5.

The spectrophotometric experiments were carried out on a Cary model-14 recording spectrophotometer. Small volume additions of inhibitor were made to the spectrophotometric cell and the resulting spectra were corrected for volume dilution.

Results

a) Inhibition by halides at pH 3.5 and 5.5 (oxygen electrode assay)

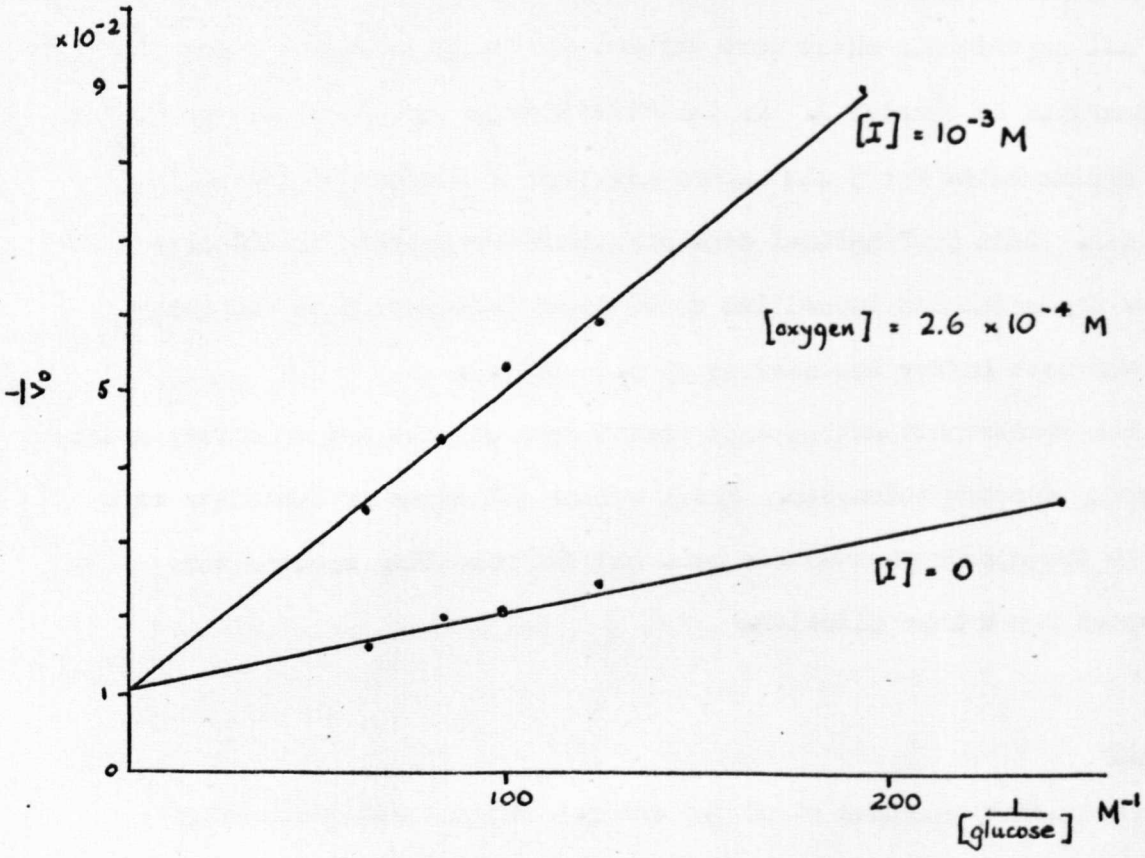
Using an enzyme concentration of 1.062×10^{-8} M, the pattern of inhibition by halides at pH 3.5 and 5.5 was investigated, and is set out in table 6.1. below.

Table 6.1. Percentage inhibition at various inhibitor concentrations.

	Concentration of inhibitor (M)			
	10^{-5}	10^{-4}	10^{-3}	10^{-2}
NaI at pH 5.5	-	-	-	2
" " pH 3.5	3	18	22	-
NaBr at pH 5.5	-	-	-	13
" " pH 3.5	16	41	57	-
NaCl at pH 5.5	-	-	-	8
" " pH 3.5	10	36	50	-
NaF at pH 5.5	-	-	-	6
" " pH 3.5	-	11	45	-

Fig.6.1. Reciprocal plots of activity inhibited by NaCl.

0·1 M-acetate buffer pH 3·5, $2{\cdot}65 \times 10^{-6}$ M-enzyme



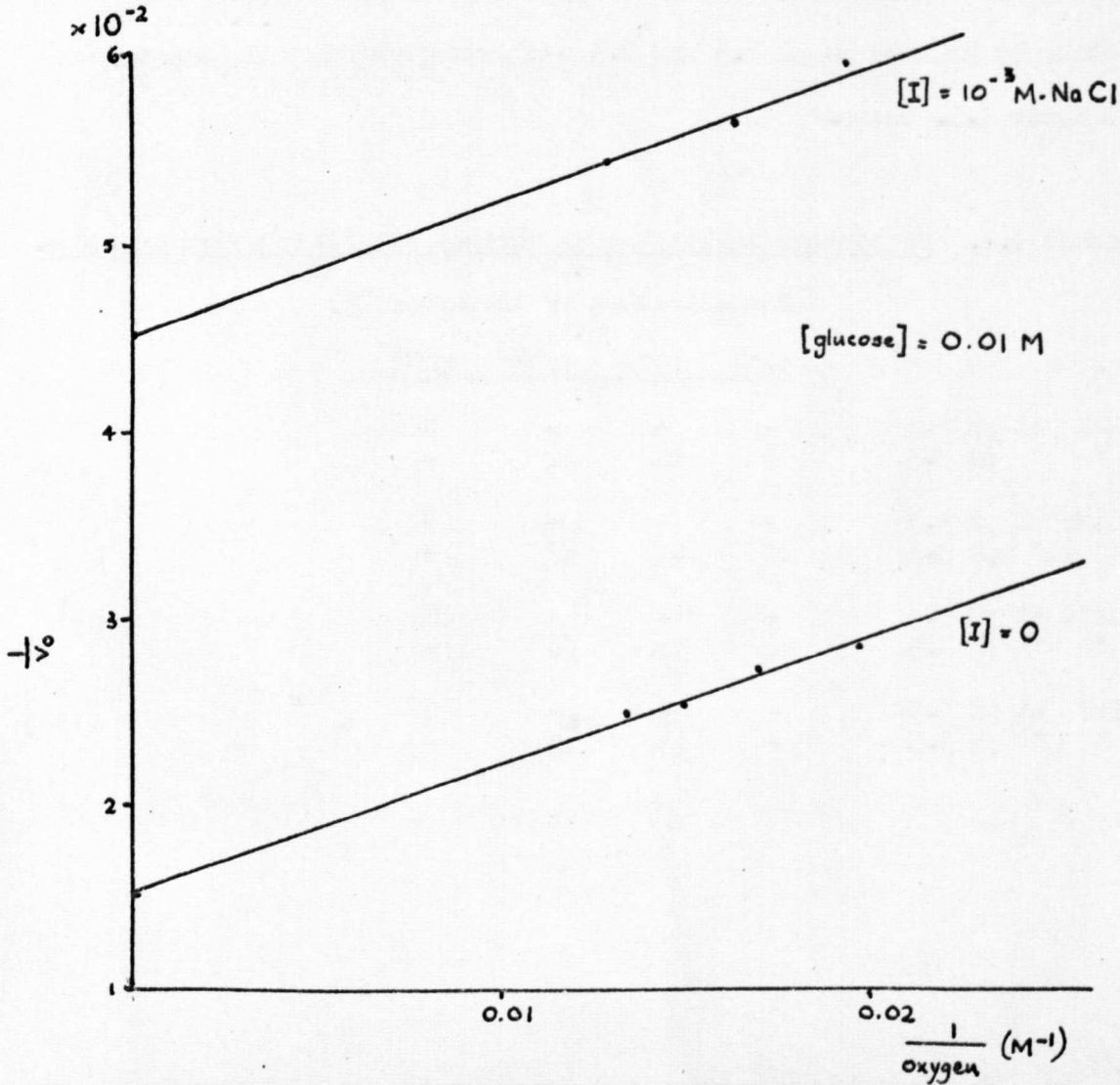


Table 6.2.

Inhibition constants for a series of anions at pH 3.5.

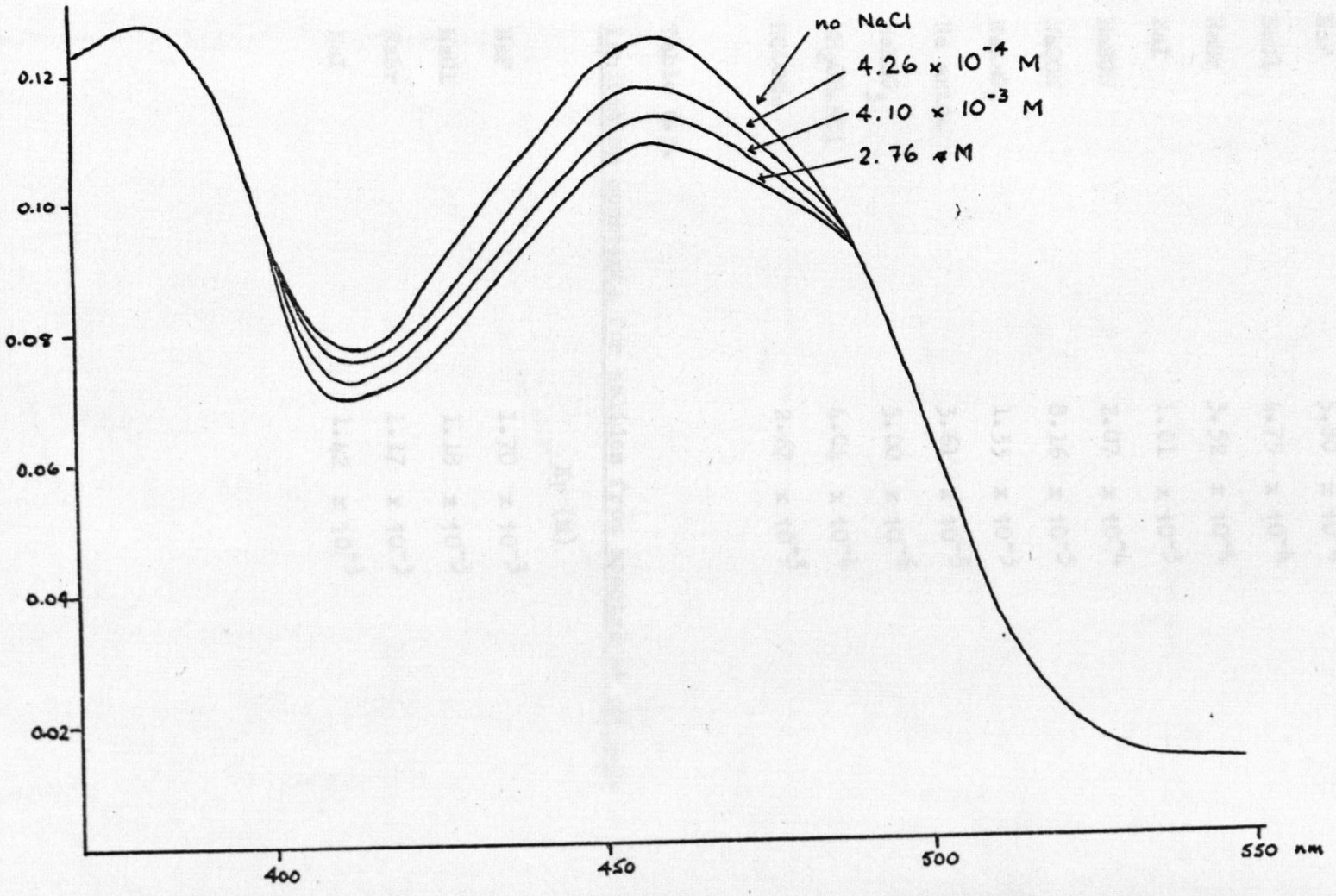
	K_I (M)
NaF	5.80×10^{-4}
NaCl	4.79×10^{-4}
NaBr	3.52×10^{-4}
NaI	1.01×10^{-3}
NaSCN	2.07×10^{-4}
NaOCN	8.16×10^{-5}
$NaAsO_2$	1.33×10^{-3}
Na azide	3.61×10^{-5}
$NaHSO_3$	5.00×10^{-6}
$NH_2OH.HCl$	4.04×10^{-4}
HCOONa	2.67×10^{-3}

Table 6.3.

Inhibition constants for halides from spectra at pH 3.5.

	K_I (M)
NaF	1.70×10^{-3}
NaCl	1.18×10^{-3}
NaBr	1.17×10^{-3}
NaI	1.42×10^{-3}

Fig.6.2. Change in part of the spectrum of glucose oxidase with NaCl.



1.097×10^{-5} M-enzyme (active site concentration) in 0.1 M-acetate buffer pH 3.5

b) Inhibition by various compounds at pH 3.5 (oxygen electrode assay).

A typical Lineweaver-Burk plot of 1/substrate concentration against 1/(rate of reaction) is shown in fig.6.1. From this the inhibition constant (K_1) for a given inhibitor was obtained and a table of such values for inhibitors at pH 3.5 is given in table 6.2. From the form of the reciprocal plots (fig.6.1) it can be seen that the inhibitor competes with glucose for the oxidised enzyme. In the reciprocal plot in which the oxygen concentration was varied, no change in slope of the line was found on addition of an inhibitor. The intercept on the ordinate of the plot (in which the glucose concentration was varied and the oxygen concentration was kept constant), indicates that the nature of the inhibition is "competitive", (see appendix 1.)

c) Investigation of halide inhibition at low pH spectrophotometrically.

A typical picture of the change in spectral pattern with additions of inhibitor is shown in fig.6.2. The K_I value for each inhibitor was obtained (see appendix 2) and these are set out in table 6.3.

Discussion

A number of anions have been shown to have an inhibitory effect on glucose oxidase activity at low pH. This includes fluoride and azide although Kusai (1960) could not show that they inhibited enzyme activity at pH 5.6. The halide ions were shown to have a stronger inhibitory effect at pH 3.5 than at pH 5.5 (see table 6.1). From the plots of reciprocal velocity against reciprocal substrate concentration, (fig.6.1.) it is clear that the halide inhibitors compete with glucose for the enzyme in its oxidised state. The may be represented as follows:-

$$E_{ox} + G \rightleftharpoons E_{ox}G \longrightarrow P_1 + E_{red}$$

$$+$$

$$I \rightleftharpoons EI$$

where E_{ox} is the oxidised enzyme, G is glucose, P_1 is D-glucono-δ-lactone, I is inhibitor and EI is enzyme-inhibitor species.

The apparent affinity of the enzyme for the inhibitor was sensitive to a lowering of pH of the reaction (table 6.1.) Thus as the enzyme became protonated the degree of binding of anionic inhibitors increased. This protonation of glucose oxidase at low pH, and subsequent reaction with halide anions has been demonstrated for P.notatum enzyme (Bright and Appleby 1969) and for A.niger enzyme (Weibel and Bright 1971). The latter authors have found that in the reductive half-reaction of glucose oxidase, the substrate glucose only combines with an unprotonated form of the enzyme. Furthermore, at low pH and in the presence of halide, the maximum turnover number is determined entirely by the rate of flavin reduction (k_2) in the reductive half-reaction, which they represented as follows:-

$$H^+E_{ox} \underset{H^+}{\overset{K_1}{\rightleftharpoons}} E_{ox} + S_1 \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E_{ox}\text{-}S_1 \xrightarrow{k_2} E_{red} + P_1$$

In the spectrophotometric experiments, addition of halide at low pH brought about a change in the 450 nm peak of the spectrum. This wavelength corresponds to FAD absorption. Thus it seems that the halide ions either interact with charged groups on the FAD molecule itself, or with charged amino-acids of the enzyme protein in the vicinity of the FAD molecule. The inhibition by bisulphite anion, described in chapter 5, was shown to involve a chemical group having a pK of 4.2. In chapter 5 reasons were discussed for suggesting that the group involved was the $-NH_2$ of the adenine of FAD. Although the addition of halides did not bleach the enzyme spectrum , it is suggested that the same group is involved in the halide binding. Weibel and Bright (1971) have presumed that the binding of halide at low pH induces a minor conformational change in the enzyme which affects both the binding of

substrate and flavin reduction. They have found that halide effects disappear at pH values greater than 6.0, which reinforces their theory of enzyme protonation at low pH.

Introduction

A systematic investigation of the effect of visible light in the presence of methylene blue dye and oxygen on amino-acids and proteins was first carried out by Weil, Gordon and Buchert (1951). Prior to this such experiments on the "photo-dynamic effect" of light and dye had been carried out on whole organisms. These experiments were extremely difficult to interpret, and they have been reviewed by Blum (1932) and Arnow (1936).

It was while using light and methylene blue dye on nicotine that Weil and Maher (1950) followed the photo-oxidative effect on particular chemical groups. They found that degradation of the aromatic (pyrrolidine) ring had occurred. They were able to correlate the extent of photo-oxidation and the wavelength of light used. This technique was used on lactogobulin (Weil and Buchert 1951), lysozyme (Weil et al 1952), chymotrypsin (Weil et al 1953), and ribonuclease (Weil et al 1955). It was shown that the technique had no effect on the viscosity or the solubility of the protein, and that it did not hydrolyse peptide bonds within the protein. However, photo-oxidation of histidine, tyrosine, tryptophan, methionine and cystine residues had occurred. When methylene blue is used as sensitiser in photo-oxidation reactions with free amino-acids, histidine, tyrosine tryptophan and methionine are oxidised at rates of the same order of magnitude as above (where they are protein-bound) in the pH range 4.0 - 9.0 (Weil et al 1951, 1952; Vodrazka, Cejka and Salak 1961). The photo-oxidation of histidine has been the subject of special investigations by Koshland and others (1958, 1960a, 1960b).

A number of dyes have been used as sensitisers in photo-oxidation experiments. Sluyterman (1962) has used proflavine (3,6-diamino acridine)

as sensitiser. In his experiments the amino-acids histidine, tyrosine, tryptophan and methionine were oxidised. He found that the pH of the experimental solution had a profound effect on the oxidation rate and on which of the four amino-acids was oxidised.

Extensive experiments on the photo-oxidation of the enzyme yeast enolase, with Rose Bengal as sensitising dye, have been carried out by Westhead (1965). He showed that loss of enzyme activity correlated with the photo-oxidation of a critical histidine residue in the protein. Brand, Gohlke and Rao (1967) have investigated the binding of Rose Bengal at the active site of liver alcohol dehydrogenase. Halcomb et al (1968) have reported the photo-oxidation of histidine residues in S-acetyl-3-phosphoglyceraldehyde dehydrogenase, using Rose Bengal, and Hoffee et al (1967) have photo-oxidised the histidine residues of rabbit muscle aldose. Methylene blue has been used as sensitiser with ribonuclease (Irie 1969), heart phosphofructokinase (Ahlfors and Mansour 1969) and aspartic aminotransferase (Martinez-Carrion et al 1967).

Though the action of the sensitising dye in photo-oxidation is not fully understood, Oster, Bellin, Kimball and Schrader (1959) have proposed a mechanism for the proflavine-sensitised oxidation of p-toluene diamine. They have suggested that the dye reacts with oxygen in the "triplet" state to give a dye-peroxide, and that this complex brings about oxidation. Blum (1932) and Sluyterman (1962) also have indicated a short-lived complex between the activated dye-oxygen intermediate and the substrate, prior to oxygen transfer. Bellin and Yankus (1968) have claimed that the photo-oxidation affects only the amino-acid aromatic side chains, as shown by the degradation products they have obtained. They have stated also that the oxidation of an amino-acid is relatively independent of the particular sensitiser used, but dependent on the pH of the reaction and on the

local environment surrounding the susceptible amino-acid residues.

Materials and Methods.

For experiments with methylene blue, the dye was made up freshly each week to a concentration of 0.2% with appropriate buffer. It was kept at 4°C and diluted with buffer as required. The sample of enzyme (2.56×10^{-6}M- active site concentration) together with 0.001% dye and 10^{-3}M-EDTA was placed in a quartz cell held in a water-jacket at 20°C. This was placed 11 cm from a 1000 watt white-light lamp from an Aldis-Rank slide projector. Water-saturated air passed over the surface of the solution in the cell to achieve continuous stirring of the solution and to reduce evaporation. Aliquots were removed at intervals during the illumination, diluted and assayed for activity using an oxygen electrode as described in chapter 2.

In the experiments with Rose Bengal, the procedure of Westhead (1965) was followed for purification of the dye. A 0.1% aqueous solution of Rose Bengal (B.D.H.,Poole) was applied to an ion-exchange column of Dowex 50(Na). The dye came straight through and was stored in the dark at 4°C. Its concentration was measured spectrophotometrically and calculated from the absorbance value of Westhead (a 0.01 mg/ml dye solution had an absorbance at 550 nm of 0.60). The sample of enzyme (8.1×10^{-6}M-active site concentration) with 5×10^{-4} mg/ml Rose Bengal was contained in 0.1 M-buffer containing 5×10^{-3}M-EDTA. Illumination was carried out as described above. Residual activity was assayed as described in chapter 2. The buffer systems used were as follows:-

pH 3.5 - 5.5 0.1 M-acetate buffer

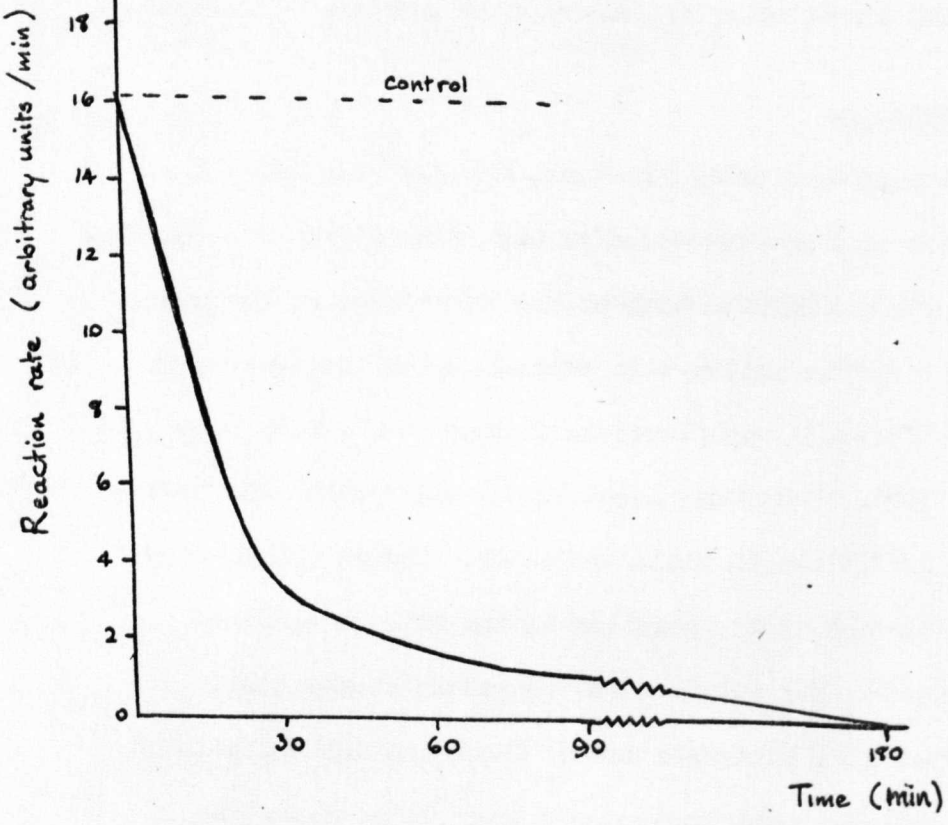
pH 6.5 - 7.5 0.1 M-phosphate buffer

pH 8.5 - 9.0 0.1 M-tricine buffer

pH 9.0 - 10.0 0.1 M-carbonate buffer

Fig.7.1. Photo-oxidation at pH 7.0 using methylene blue.

0.7×10^{-5} M-enzyme, 0.1 M-phosphate buffer



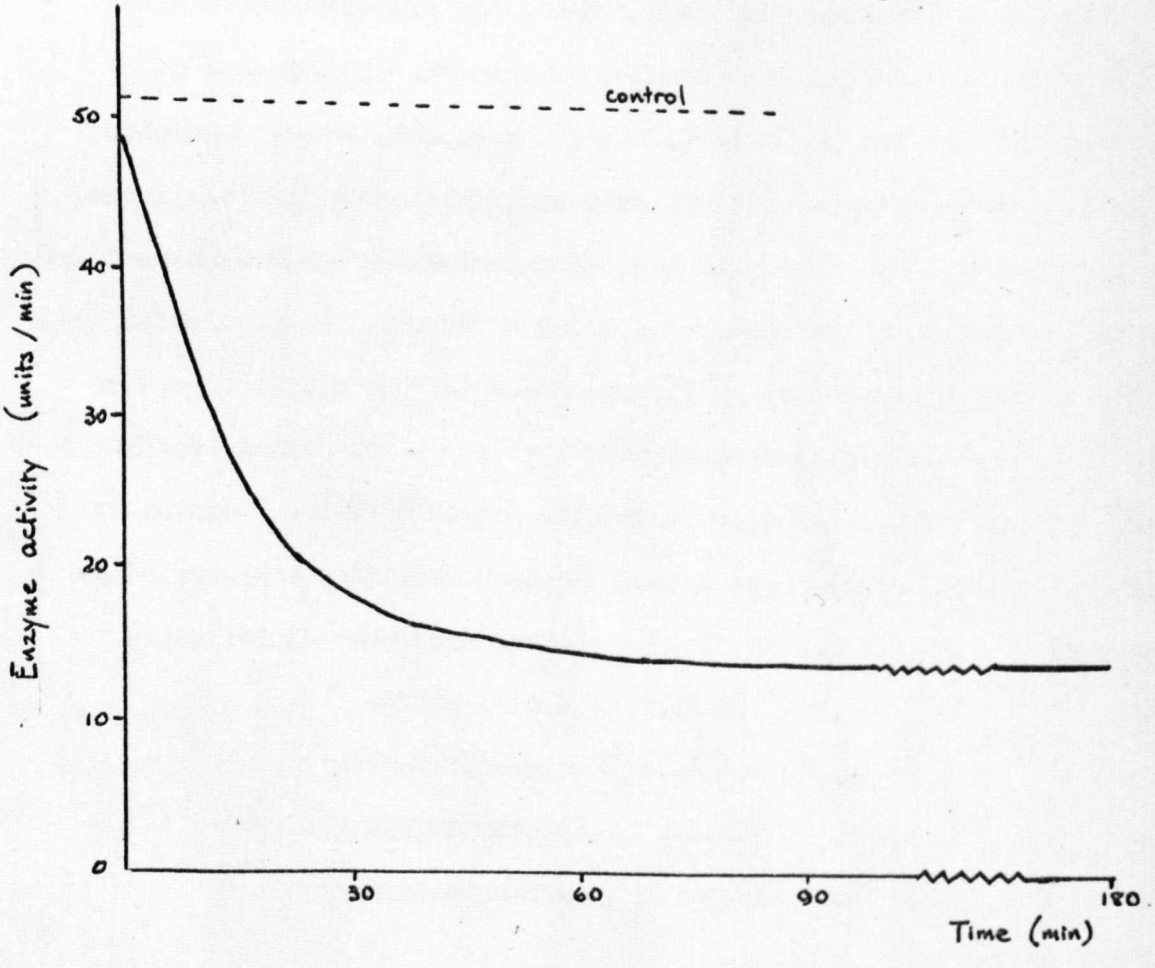


Fig.7.2. Photo-oxidation at pH 7.0 using Rose Bengal.

0.7×10^{-5} M-enzyme, 0.1 M-phosphate buffer

Fig.7.3. First order plot of the reaction shown in fig.7.1.

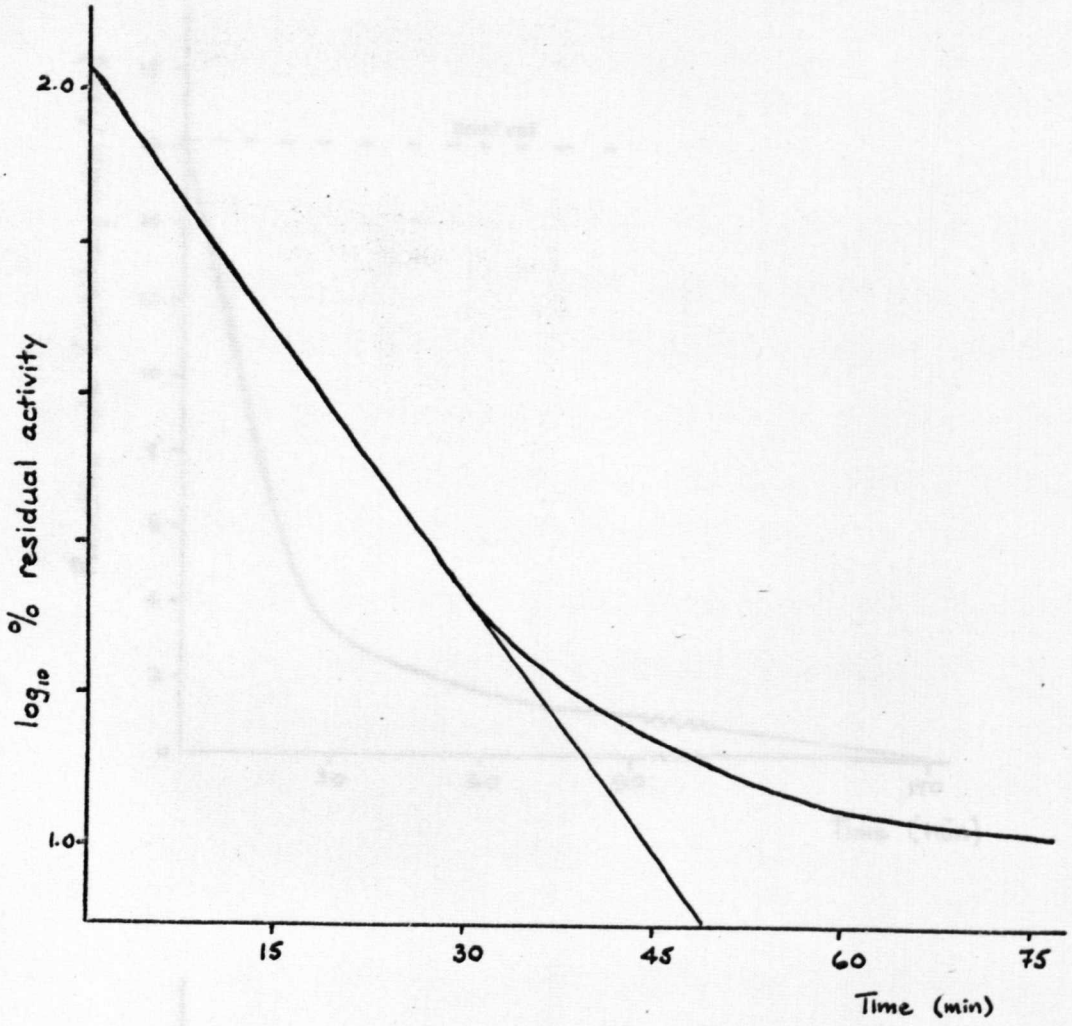
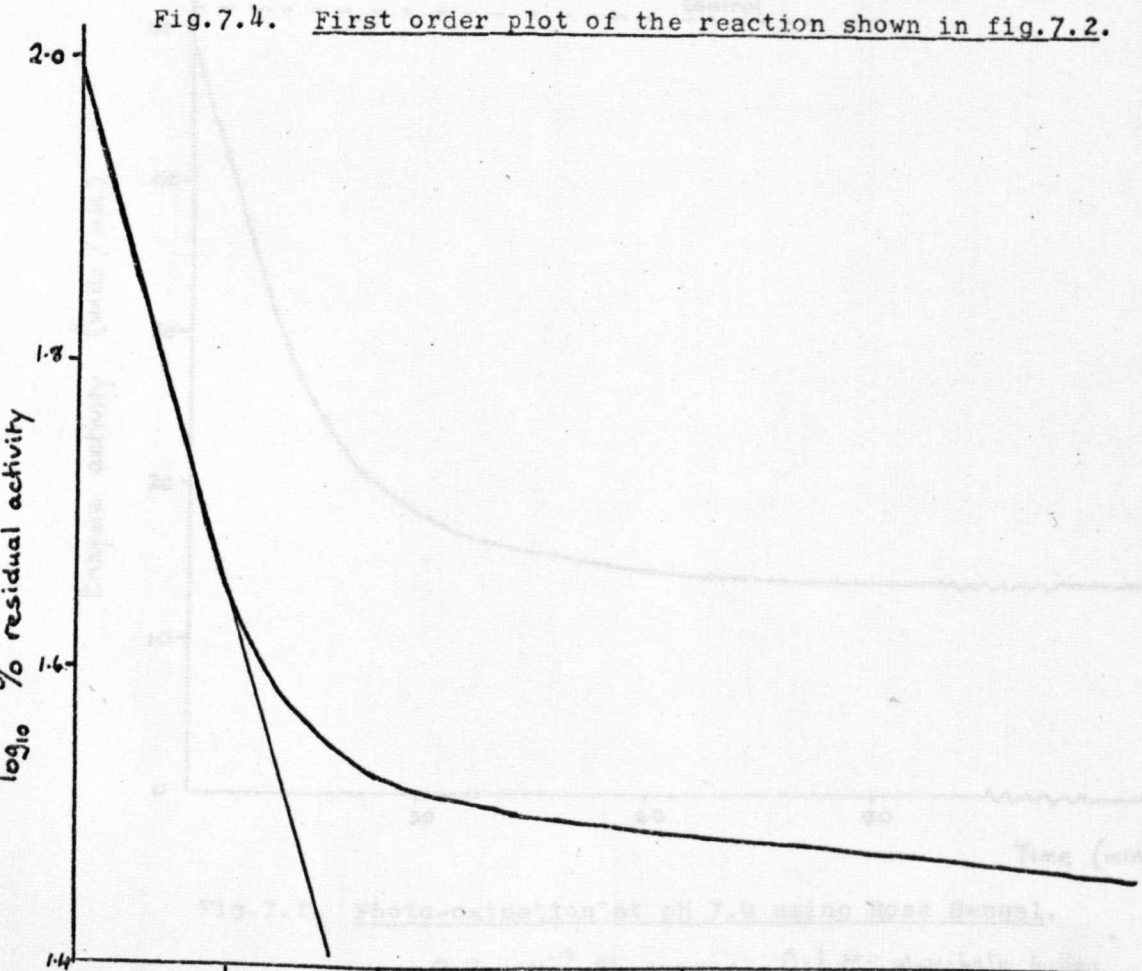


Fig.7.4. First order plot of the reaction shown in fig.7.2.



Results.

1. Effect of photo-oxidation at pH 7.0 with either methylene blue or Rose Bengal as sensitiser.

The loss of activity with time of illumination is shown for each sensitiser in figs. 7.1 and 7.2. From the shape of the curves the initial loss of activity seemed to follow first order kinetics, for which the equation is :

$$\log_e \frac{a}{(a - x)} = kt \qquad 1.$$

where a is the initial activity, $(a - x)$ is the residual activity at time t, k is the first order rate constant. This can be plotted as the logarithm of the percentage residual activity against time, when

$$\log_{10}\left[\frac{(a - x)}{a} \times 100\right] = -\frac{kt}{2.303} + \log_{10} 100$$

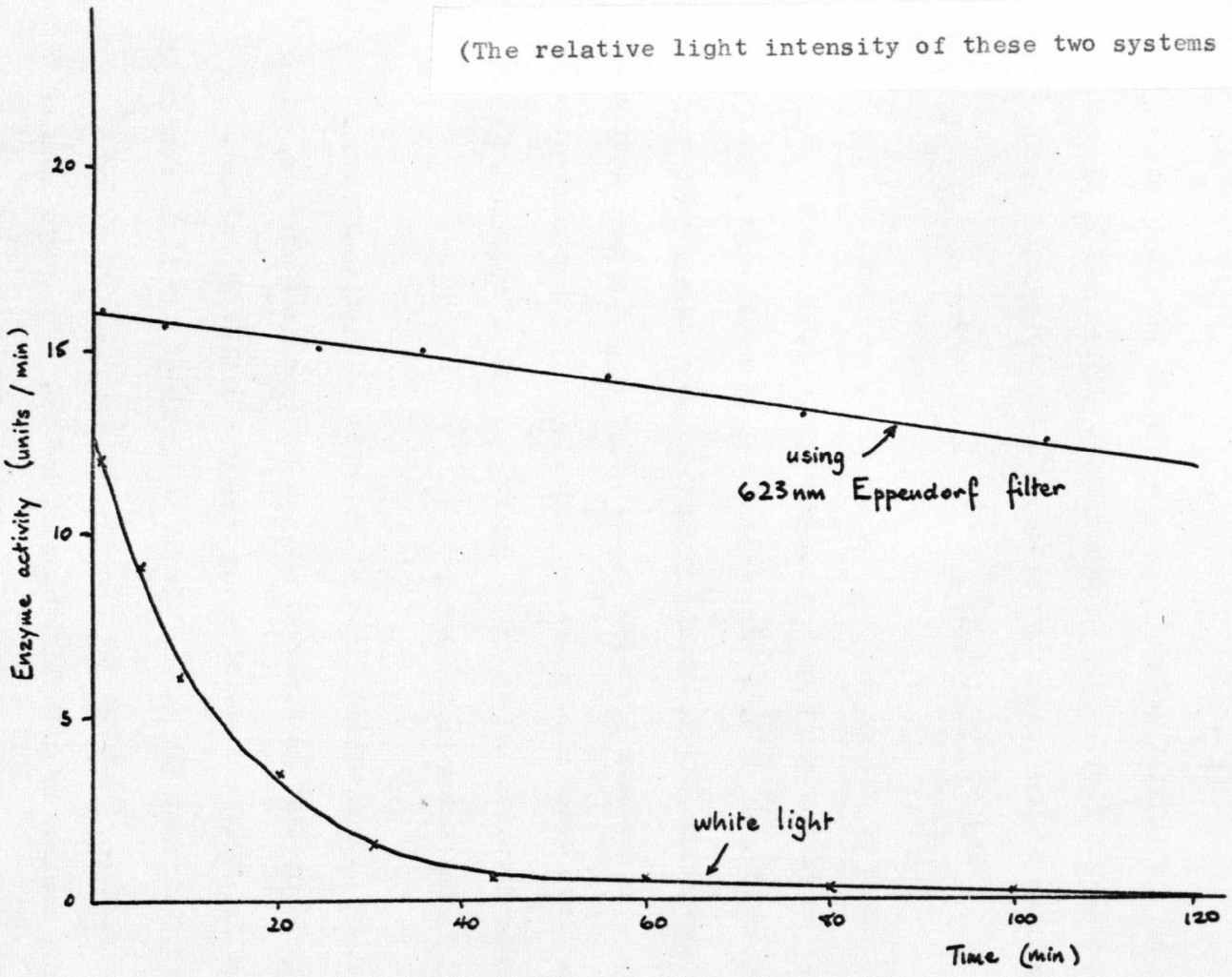
From the slope of the graph k may be obtained, as shown in figs 7.3 and 7.4. For methylene blue $k = 0.054\ min^{-1}$, and for Rose Bengal $k = 0.042\ min^{-1}$, at pH 7.0. In control experiments containing dye but not exposed to light, no significant loss of enzyme activity was observed over the same period of time. Experiments showed that k was a pseudo first order rate constant, dependent on dye concentration, oxygen concentration and on light intensity, all of which were kept constant in the above experiments.

2. Effect of monochromatic light on the rate of photo-oxidation.

Only light that is absorbed can produce chemical changes, which therefore is limited to wavelengths absorbed by the sensitiser. An Eppendorf filter at 623 nm was placed in front of the light source in this experiment. This was the closest available filter to the absorption maximum of methylene blue at 668 nm. The comparison of photo-oxidation at this specific wavelength with that of white light is shown in fig.7.5. At the end of a two hour period, when the sample

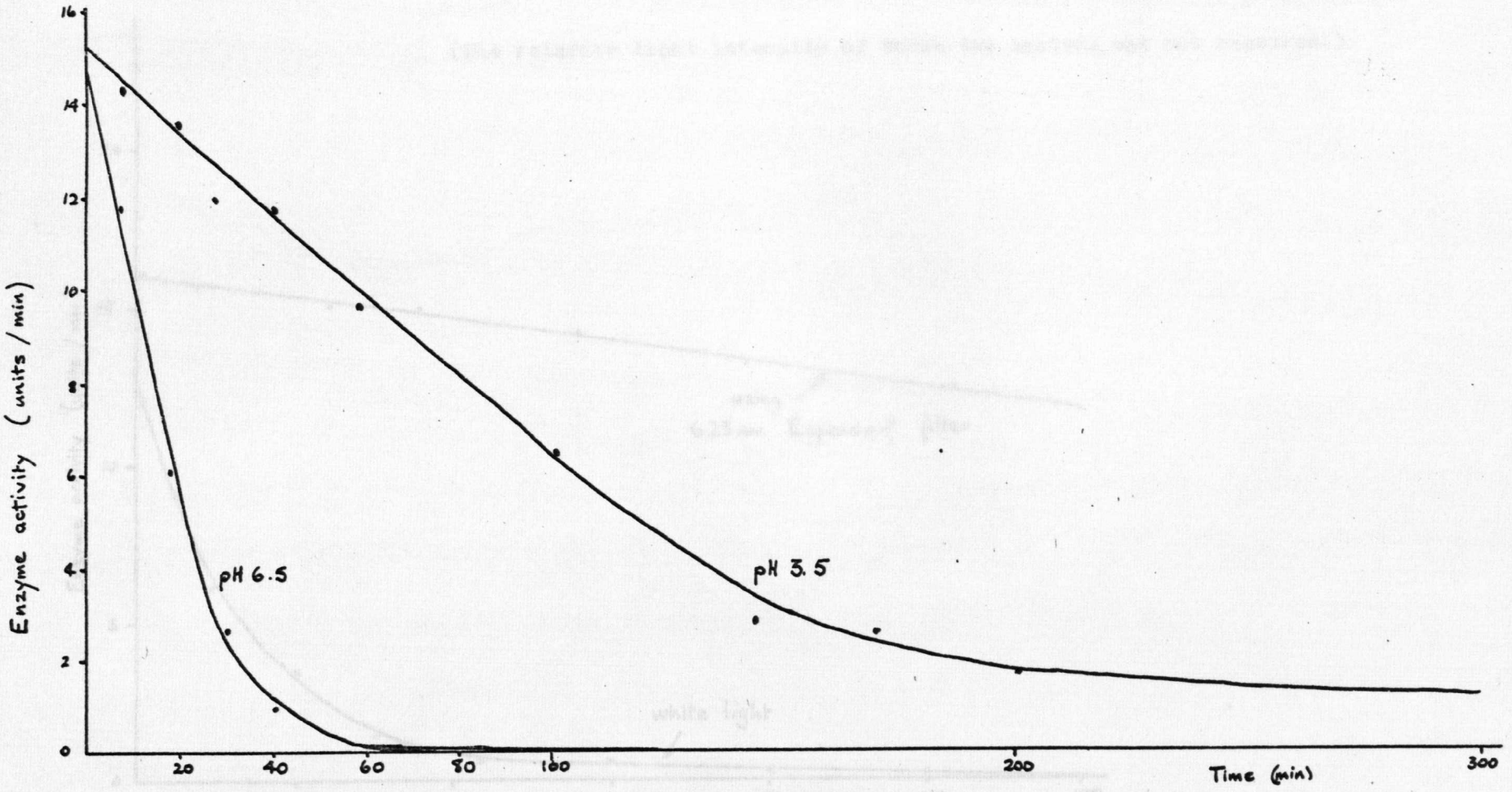
Fig.7.5. Effect of wavelength on photo-oxidation.

(The relative light intensity of these two systems was not measured.)



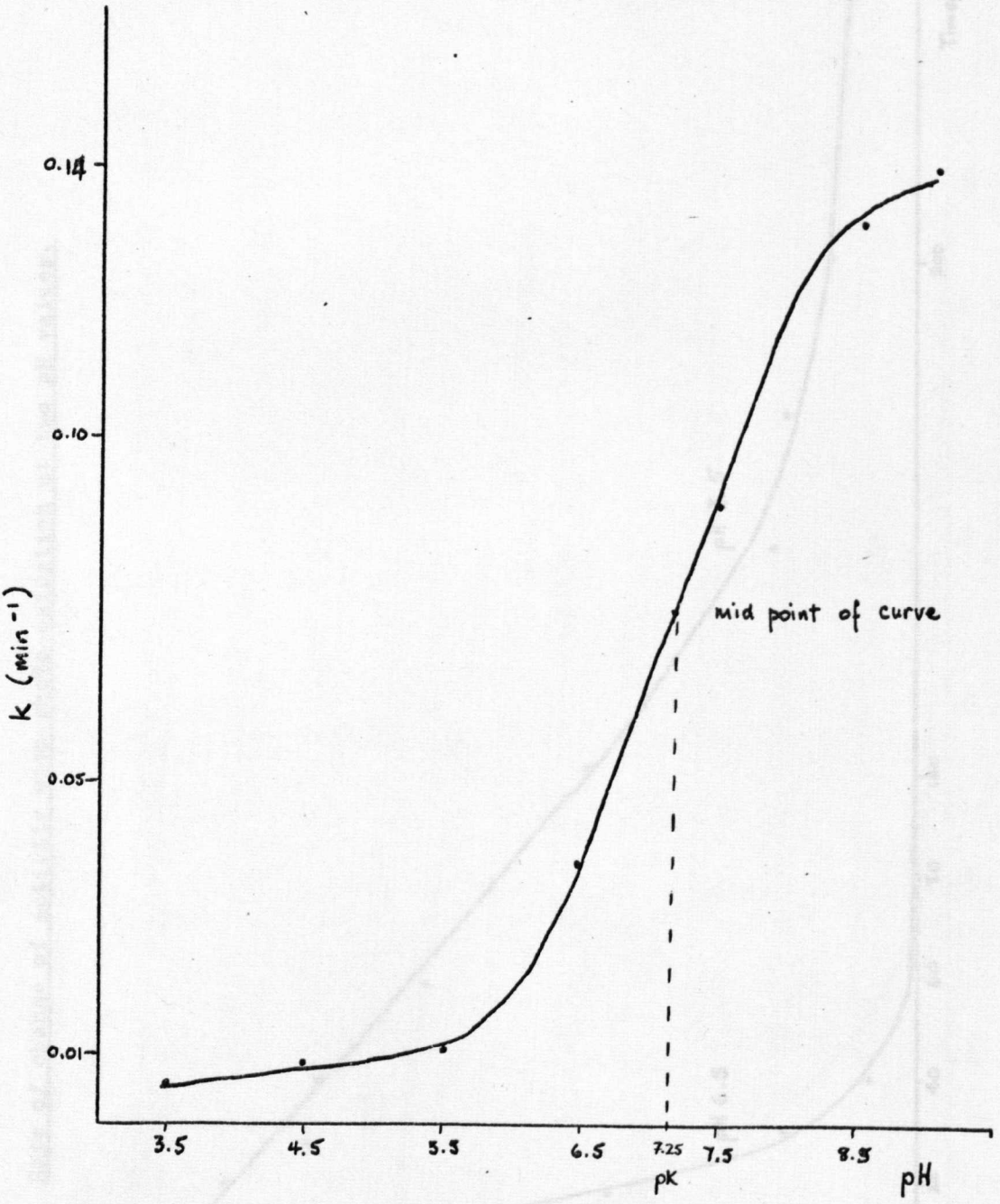
2.56.× 10^{-6} M-enzyme in 0.1 M-phosphate buffer pH 7.0 with 0.001% methylene blue and 10^{-3} M-EDTA

Fig.7.6. Rate of change of activity with photo-oxidation at two pH values.



2.56×10^{-6} M-enzyme in 0.1 M buffer of appropriate pH, 0.002% methylene blue, 10^{-3} M-EDTA

Fig. 7.7. Dixon plot of the change in rate constant with pH.



exposed to white light had lost all of its activity, the sample exposed to light at 623 nm had lost only 24% activity. No light intensity measurements were made.

3. Effect of pH on photo-oxidation.

Using methylene blue (0.002%) as sensitiser, photo-oxidation was carried out at pH 3.5, 4.5, 5.5, 6.5, 7.0, 7.5, 8.5, 9.0. The rate of change of activity with pH is illustrated in fig.7.6. The graphs were replotted according to the first order rate equation (equation 1 above) and k values were obtained. These are shown in table 7.1 and graphically in fig.7.7. By taking the midpoint of the curve, the pK of the ionising species is obtained (Dixon 1953, 1964). From this graph the pK was 7.2.

Table 7.1.

Values of k obtained at various pH values.

pH	Mean values of k (min^{-1})
3.5	0.0056
4.5	0.0082
5.5	0.0120
6.5	0.0410
7.0	0.0610
7.5	0.0710
8.5	0.1320
9.0	0.1370

It will be seen from fig 7.8. that the change in activity of the enzyme with pH showed the same pattern for different periods of illumination. The photo-chemical effect is dependent on pH and shows maximal effect at high pH values.

Fig.7.8. Effect of pH on photo-oxidation.

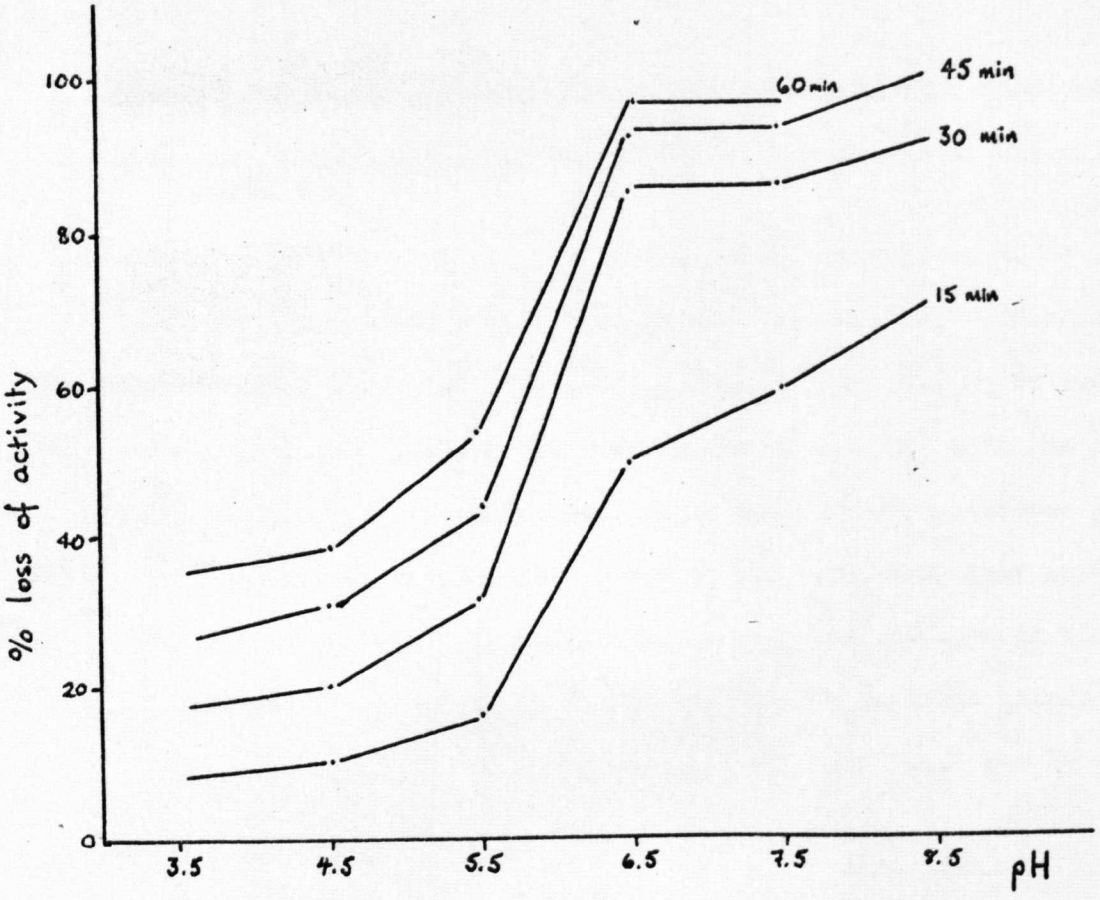
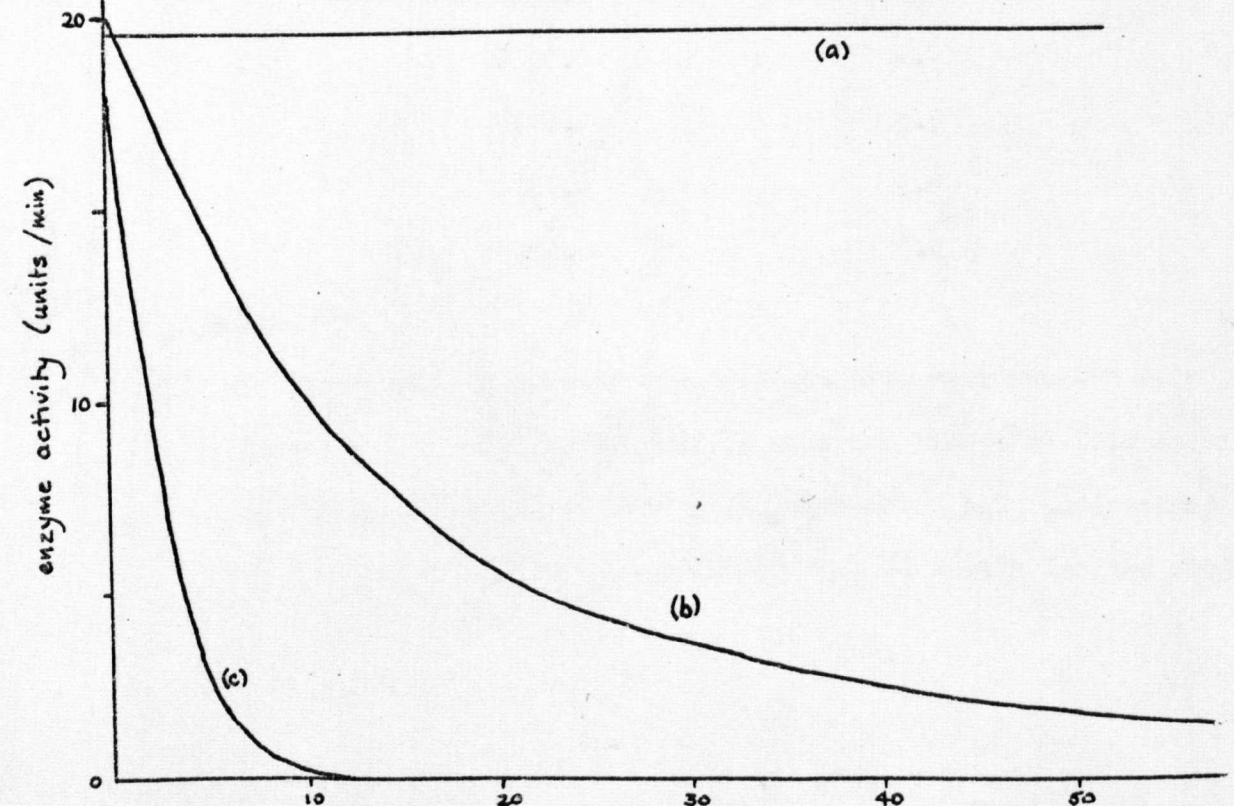


Fig.7.9. Enzyme at pH 9.0 with or without illumination, or methylene blue (described in text).



4. To investigate the loss of activity with illumination at pH 9.0 and 10.0.

Using methylene blue and freshly made carbonate buffer, three experiments were performed.

a) No dye and no illumination.

b) No dye but with illumination

c) With dye and illumination.

The results are shown for pH 9.0 in fig.7.9.

Photo-oxidation in the presence of a sensitising dye proceeds rapidly, with total loss of activity after 12 min. No loss of activity was obtained in the absence of both light and dye, but in experiment (b) with no dye, loss of activity occurred as a function of time. After one hour 93% loss of activity had been obtained, indicating that some photo-chemical change had taken place in the enzyme which did not require a dye-sensitiser. Oxygen had been present in these experiments. No tests were made at this stage to see if the photo-chemical effect required oxygen.

Discussion.

Both methylene blue and Rose Bengal were satisfactory sensitisers in the photo-oxidation experiments carried out on glucose oxidase. In either case the reaction initially followed first order kinetics. However the pseudo first order rate constant varied with pH. It has been suggested (e.g. Weil et al 1951) that the mechanism of dye-sensitised oxidation is as follows:-

$$\text{amino-acid} + \text{dye} \xrightleftharpoons{h\nu} \text{dehydrogenated amino-acid} + \text{reduced dye}$$

$$\text{reduced dye} + O_2 \rightleftharpoons \text{dye} + H_2O_2$$

$$\text{dehydrogenated amino acid} + H_2O_2 \rightleftharpoons \text{degradation products}.$$

With methionine as the amino-acid in question, the hydrogen peroxide plays a part in the destructive process. It is not certain whether this is the case with tyrosine, tryptophan, histidine or cystine. If the above mechanism is correct, then the reaction of dye and amino-acid must follow first order kinetics.

It has been found that the rate constant k is very sensitive to pH, and the plot of k versus pH indicated that one of the reacting species had a pK of 7.2. Methylene blue has a pK of 11.6 and Rose Bengal a pK of 4.3 - 4.5 (Bellin and Yankus 1968). Therefore the pK of 7.2 must be that of an ionising group in the enzyme, which is being oxidised by the light/dye reaction. Of amino-acids known to be oxidised in this way, only the histidine imidazole group has a pK in this region i.e. pK 5.6 - 7.0 (25°C). Weil, Gordon and Burchert (1951) have shown that photo-oxidation of histidine involves only the imidazole ring. For the free histidine, they suggest that photo-oxidation causes the rupture of the imidazole ring to give an aldehyde group which forms an internal Schiff base.

In the experiment with monochromatic light it was found that placing a filter (Eppendorf 623 nm) in front of the light source greatly reduced the photo-chemical effect. This may have been due to a reduction of the incident light, or that the wavelength chosen was too specific for the reaction.

In experiment (4) it was found that in the absence of sensitiser, considerable loss of enzyme activity occurred when the enzyme was exposed to light at high pH and in the presence of EDTA (present in all the experiments in this chapter). Such a phenomenon has been described and utilised by McCormick, Koster and Veeger (1967). Further experiments using this photo-chemical reaction on P. amagasakiense glucose oxidase are described in the next chapter. It is not possible

that photo-destruction of this type occurred at low pH, since the loss of activity in the presence of dye was very slow. Experiments on photo-oxidation at high pH were masked by the photo-destructive effect. The role of oxygen in this effect will be described in the next chapter.

The binding of Rose Bengal to an enzyme has been investigated by Brand, Gohlke and Rao (1967) using liver alcohol dehydrogenase. They measured the fluorescence excitation-emission spectra of free and bound dye (i.e. dye in the presence of enzyme). An enhancement of dye fluorescence in the presence of enzyme was observed, together with a small shift of emission maximum from 562 to 570 nm. Titration of Rose Bengal into phosphate buffer gave values of free dye fluorescence as a function of concentration. Titration of dye with alcohol dehydrogenase provided corresponding values for the bound dye. From an analysis of these values using Scatchard plots, an association constant of $3.4 \times 10^{-5}\ M^{-1}$ and two binding sites for dye per enzyme molecule were obtained.

CHAPTER EIGHT THE PHOTO-CHEMICAL REACTION.

Introduction.

As mentioned previously, in the presence of EDTA at high pH, glucose oxidase lost its activity with increasing exposure to white light. Massey and Palmer (1966) have used this photo-chemical reaction (not at high pH) to quantitatively produce flavoprotein semiquinones. This does not normally result in photo-destruction, which was obtained with glucose oxidase at high pH. Therefore this is either a) a different type of phenomenon, or b) possibly the flavin semiquinone undergoes some secondary reaction at high pH, which results in a loss of activity.

McCormick, Koster and Veeger (1967) have investigated the mechanism of this photo-chemical reaction (reduction) of FAD and FAD-dependent enzymes. They have shown that the rate of photo-reduction of D-amino acid oxidase > L-amino acid oxidase > glucose oxidase > oxynitrilase. Addition of free flavin to the reaction markedly enhanced photo-reduction of those enzymes having readily dissociable FAD. Urea was also found to enhance photo-reduction while increased temperature did not have a great effect. Hemmerich, Nagelschneider and Veeger (1970) have speculated on the role of a histidine residue in the enzyme protein which stabilises the semiquinones produced by glucose oxidase on half-reduction in the absence of substrate.

Materials and Methods.

All fluorimeter experiments were performed using a Farrand mark II spectrofluorimeter (Farrand Optical Company Inc.,) at 25°C. The intensity of FAD fluorescence was measured at 520 nm using monochromatic light at 450 nm for excitation. Protein fluorescence (due to tryptophan residues in the protein) was measured at 350 nm using an excitation wavelength of 295 nm. In experiments involving the use of a fixed

wavelength, the excitation lightpath of the fluorimeter was used. Enzyme activity was assayed as described in chapter 2.

For anaerobic experiments, nitrogen gas was bubbled for 30 min. through all solutions except that of enzyme, to flush out the air. The experiments were carried out in specially constructed fluorimeter cells shown below. When filled with the appropriate solutions in the main cell and enzyme in the side arm, the cell was evacuated at a water pump to a pressure of 1 cm mercury. The pressure was released under nitrogen, and the whole treatment of evacuation repeated. The cell was kept at $4^{o}C$ throughout the evacuation procedures. The cell was wrapped in foil to exclude light from the solutions where necessary. Evacuation at the water pump took about 1 hour in all; in a control experiment, after $1\frac{1}{2}$ hours at the pump, a loss of 10% cell contents was measured.

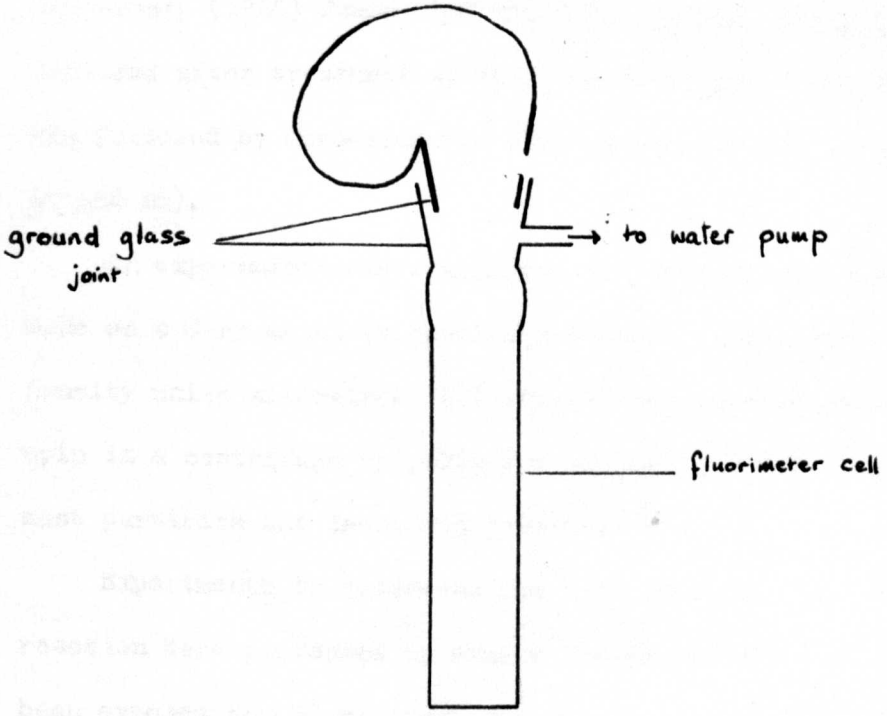


Diagram of the special fluorimeter cells used.

Polarization of fluorescence was carried out as described by Swoboda (1969a). The intensity of fluorescence was always measured at the four settings of the polarizer and analyser. The polarization of fluorescence

was defined as

$$p = \frac{V_v - KH_v}{V_v + KH_v}$$

by Weill and Calvin (1963) where H_v setting refers to a setting of the polarizer so that the electrical vector of the polarized light produced is vertical to the plane of that of the analyser. $K = V_h/H_h$ is a correction factor for the non-ideal transmission of polarized light through the mono-chromators. The intensity of fluorescence was measured at each of the four settings in turn before and after illumination. The polarization of fluorescence was calculated for before and after illumination.

Release of total flavin from the enzyme was achieved by holding the enzyme at 90-95°C for 10 min in a dark, stoppered tube. Swoboda and Massey (1965) found that the total flavin of A. niger enzyme was released after treatment at 100° for 2-10 min. The release of flavin was followed by measuring the fluorescence at 520 nm (excitation at 450 nm).

In experiments where spectra were obtained measurements were made on a Cary model-14 spectrophotometer, using the 0 - 2.0 optical density units slidewire. All enzyme samples were subjected to a hard spin in a centrifuge (50,000g for 60 min) to clear the solution of dust particles and denatured protein.

Experiments to determine the rate constants for the enzyme reaction were performed on enzyme samples before and after they had been exposed to 450 nm light for 60 min, at pH 10.0 with EDTA present. The enzyme samples were assayed for glucose oxidase activity as described in chapter 2, and the curves of oxygen consumption were analysed according to the integrated rate equation. The integrated rate equation is derived as follows:-

The steady state rate equation for glucose oxidase expressed by Bright and Gibson (1967) and in Dalziel notation (Dalziel 1957) is

$$\frac{e}{v_o} = \phi_o + \frac{\phi_1}{S_1} + \frac{\phi_2}{S_2}$$

The initial velocity $v_o = \left(\frac{dP_2}{dt}\right)_o$, S_1 and S_2 are the concentrations of glucose and oxygen respectively, P_2 is the hydrogen peroxide concentration, e is the enzyme concentration, ϕ_o , ϕ_1 , ϕ_2 are the velocity constants. The oxygen concentration at time t is $(S_2)_t = (S_2)_o - (P_2)_t$.

Assuming (1) that $S_1 >> S_2$, then $(S_1)_t \simeq (S_1)_o$ and that $S_2 >>$ e

(2) that there is no back reaction

(3) that there is no inhibition by hydrogen peroxide or glucono-δ-lactone.

as $v_t = \left(\frac{dP_2}{dt}\right)_t$

then $e\,\frac{dt}{dP_2} = \phi_o + \frac{\phi_1}{(S_1)_o} + \frac{\phi_2}{(S_2)_o - (P_2)}$

which on integration gives

$$\frac{et}{(P_2)_t} = \phi_o + \frac{\phi_1}{(S_1)_o} + \frac{\phi_2}{(P_2)_t}\left(\ln \frac{(S_2)_o}{(S_2)_o - (P_2)_t}\right)$$

The three assumptions above have been subjected to experimental test and have been found to be reasonable for glucose oxidase.

A plot of $\frac{et}{(P_2)_t}$ against $\frac{1}{(P_2)_t}\left(\ln \frac{(S_2)_o}{(S_2)_o - (P_2)_t}\right)$ gives a slope = ϕ_2 and an intercept = $\phi_o + \frac{\phi_1}{(S_1)_o}$

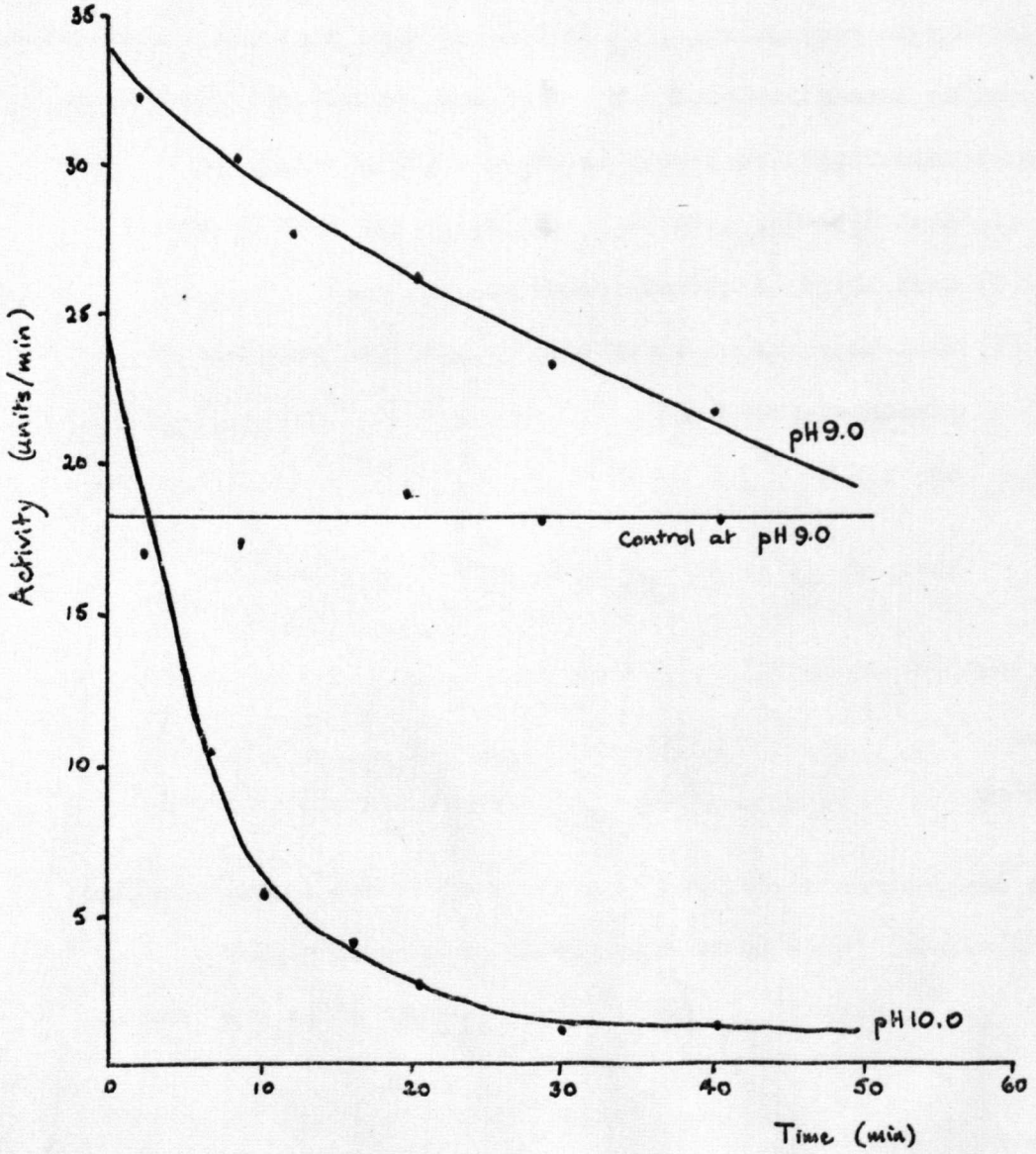
A second plot of the intercepts from the first plot against $1/(S_1)_o$ has a slope of ϕ_1 and intercept of ϕ_o .

Results

1. The effect of light at 450 nm on the photo-chemical reaction.

This experiment was a continuation of experiment (4) in chapter 7. On the principle that only light that is absorbed can produce chemical

Fig.8.1. Photo-oxidation at 450 nm, no dye.



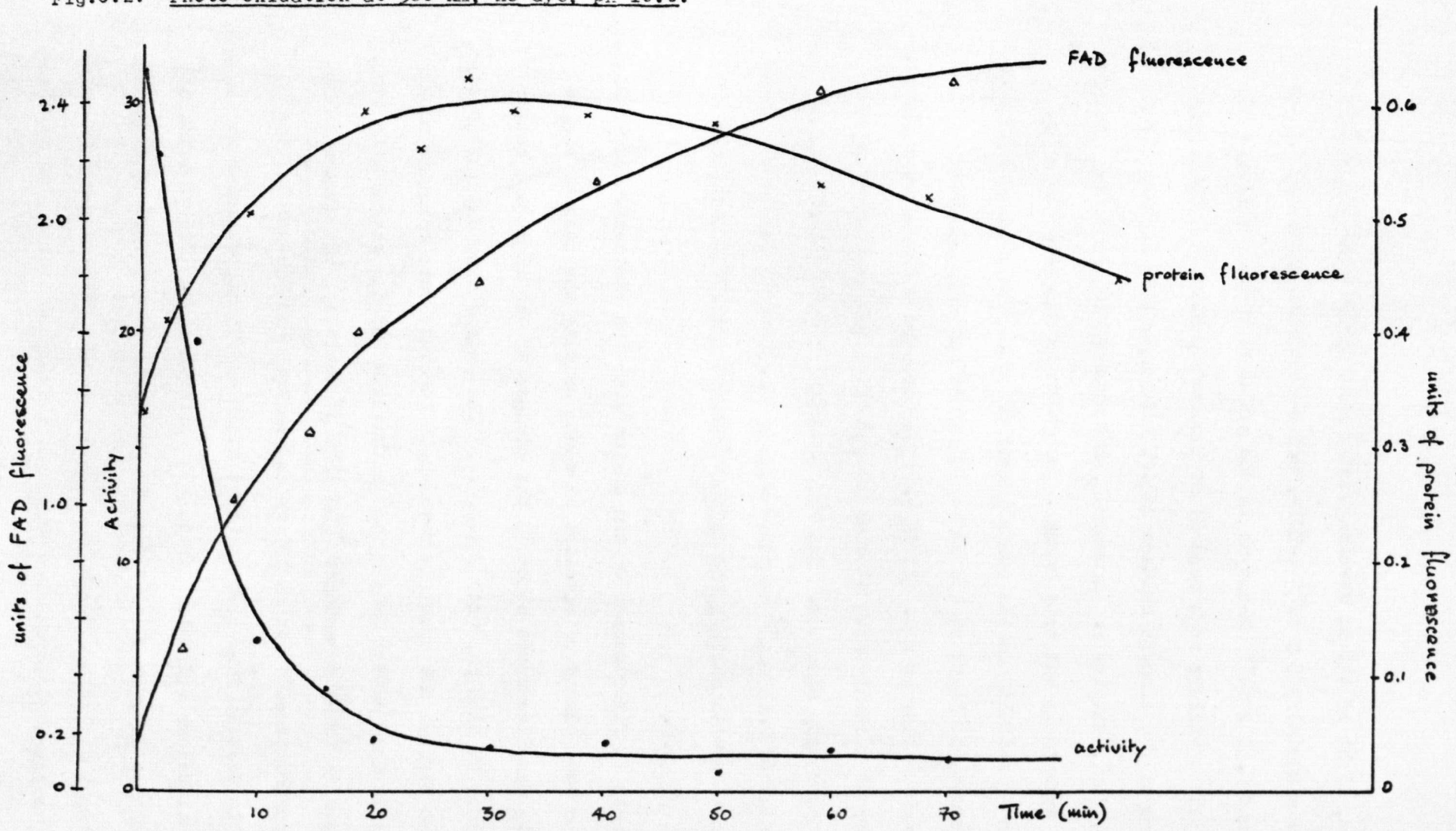
The reaction mixture was 5×10^{-3} M-EDTA, 0.1 M-carbonate buffer and 8.19×10^{-6} M-enzyme in a ratio of 1:3:1. It was illuminated by monochromatic light at 450 nm.

changes, light of 450 nm wavelength was used to illuminate a reaction mixture containing 5×10^{-3} M-EDTA, 0.1 M-carbonate buffer pH 9.0 or 10.0, and 8.19×10^{-6} M-enzyme in the ratio of 1:3:1. Aliquots of the reaction mixture were removed at intervals, diluted with buffer and assayed for glucose oxidase activity as described in "Materials and Methods", chapter 2. A control, not exposed to light, was set up and assayed in the same manner. As shown in fig.8.1. there was no loss of activity in the control over 60 min. The enzyme at pH 10.0 when exposed to light at 450 nm rapidly lost activity and was inactivated after 40 min. At pH 9.0 inactivation occurred more slowly. The loss of activity with time of illumination was plotted using the first order rate equation. The rate constant k (at pH 10.0) had a mean value of 0.13 min^{-1} compared with $k = 0.88$ min^{-1} for illumination with white light in the presence of 0.001% methylene blue at pH 10.0.

2. Changes in fluorescence of the enzyme with the photo-chemical reaction.

The possibility of alkaline pH alone causing the loss of enzyme activity was discounted since in the absence of the light the enzyme was stable to high pH, (see fig.8.1.). The possibility existed however, that the high pH and specific wavelength together were bringing about changes in the protein leading to inactivation of the enzyme with time. Therefore in these experiments the loss of activity, the change in FAD fluorescence (excitation at 450 nm and emission at 520 nm) and the change in protein fluorescence (excitation at 295 nm, emission at 350 nm) were followed as a function of time of illumination. The experimental solution was described in (1) above, and three experiments were performed using light at 450 nm, 380 nm and 320 nm. These wavelengths were chosen as they correspond to the two peaks and a trough of the enzyme spectrum

Fig.8.2. Photo-oxidation at 380 nm, no dye, pH 10.0.



respectively. Fig.8.2. shows the results using light at 380 nm. The results at 450 and 320 nm showed a similar pattern of enzyme inactivation with time, and an increase in both FAD and protein fluorescence which tended to level off with time. The rate of increase of fluorescence varied from one experiment to another at all wavelengths.

The experiments seemed to indicate that the protein was being modified and that free FAD, which has a greater fluorescence than protein-bound FAD, was being released. Polarization experiments were performed in order to show that the fluorescent FAD was no longer protein-bound.

3. The change in polarization of FAD fluorescence.

The binding of a small molecule such as FAD, to a macromolecule greatly reduces its Brownian rotational diffusion (Swoboda 1969a). Therefore if FAD was being released from the slightly fluorescent FAD-protein complex a change in polarization of FAD fluorescence should be seen. The results of such measurements are shown in table 8.1., which were made before and after illumination at 450, 380 and 320 nm as in experiment (2) above.

Table 8.1. Polarization of fluorescence measurements.

	Polarization of fluorescence
Before illumination (450 nm)	0.029
After " "	0.040
Before illumination (380 nm)	0.029
After " "	0.023
Before illumination (320 nm)	0.043
After " "	0.034

From the results in table 8.1. it can be seen that there was no change

in polarization of fluorescence on illumination of the enzyme. Swoboda (1969a) has shown that the polarization value for the FAD-protein complex of A. niger is 0.3, while that for the free FAD is 0.03 - 0.04. Therefore in the above experiments there must have been some free FAD present throughout, whose fluorescence masked that of the protein.

4. To investigate whether any free FAD was present in the enzyme sample.

The fluorescence of both the FAD and the protein of the enzyme sample was measured before and after heat treatment (described in the methods section). It was assumed that all the flavin had been released from the protein by the heat treatment and one could therefore measure the total fluorescence of the FAD. 24% of the total fluorescence was obtained from the enzyme sample before heat treatment. In the FAD-protein complex, FAD fluorescence is almost totally quenched (Swoboda and Massey 1966, Swoboda 1969a). Thus the high fluorescence obtained from the untreated enzyme must indicate a considerable amount of free flavin present.

5. Photo-chemical experiments to discover the conditions of photo-destruction.

The experimental solutions all contained 1.7×10^{-5} M-enzyme, 10^{-2} M-EDTA, 0.1 M-carbonate buffer, pH 10.0, and water in the ratio of 1:2:6:1. This mixture was exposed to light at 450 nm in the presence and absence of air for a fixed period of 45 min. The enzyme mixture was sampled for activity before and after the 45 min period as described in "Materials and Methods" chapter 2, as sampling during the progress of anaerobic experiments was not possible. A control was made anaerobic and kept dark.

The effect of air on photo-chemical effects was investigated by exposing to the light enzyme mixture under reduced pressure and containing nitrogen (called anaerobic), and an enzyme mixture in

equilibrium with the air. The results are shown in table 8.2.

Table 8.2. Photo-chemical effect in the presence and absence of air.

	Mean residual enzyme activity (arbitrary units)
Before illumination	30.5
After 45 min aerobic exposure to light	3.0
" " anaerobic " " "	1.5

Thus the photo-chemical events taking place were insensitive to the presence or absence of oxygen.

The possible protective effect of the substrate glucose was tested. The above experiment was repeated with 10^{-2} M-glucose substituted for the water. The control had no glucose but water and was not at reduced pressure. The results are in table 8.3.

Table 8.3. Effect of substrate protection.

	Mean residual enzyme activity (arbitrary units)
Before illumination	31.5
After 45 min illumination	2.35
" " " with 10^{-3} M-glucose	31.7

In the presence of glucose, no loss of enzyme activity with illumination was obtained. This protection by the substrate indicated that it was the oxidised enzyme species which was being affected by the light in the absence of glucose. To confirm this a further experiment was set up in which dithionite was added to the enzyme mixture instead of the water, to a final concentration of 10^{-3}M. Dithionite ($S_2O_6^{2-}$) is a noted powerful reducing agent, which readily converts the oxidised enzyme to the fully reduced state. It might have been expected that no loss of activity would occur in the

presence of dithionite if, as has been suggested, only the oxidised enzyme was sensitive to light under the experimental conditions. It was found however that inactivation of the enzyme did occur, although the assays for activity were affected by the dithionite which reduced the molecular oxygen in the assay solution, because of the design of the experiment.

Excess dithionite was removed from subsequent experiments in the following way. At the end of the period of illumination, the cells were opened to the atmosphere and glucose added to a concentration of 10^{-2}M. Addition of glucose caused the enzyme to "turn-over" to the oxidised state prior to the assay, and the remaining dithionite was oxidised by the air and did not interfere with the assay. The results showed that while the dithionite reduced and inactivated the enzyme, glucose addition restored the enzyme activity to samples which had not been illuminated (i.e. the controls). Exposure to light inactivated the enzyme irreversibly whether or not the enzyme solution contained dithionite.

6. The rate constants before and after illumination.

In this experiment the velocity constants for the enzyme, described by Dalziel (1957) were calculated. The rate equation may be described as

$$\frac{e}{v_o} = \phi_o + \frac{\phi_1}{S_1} + \frac{\phi_2}{S_2}$$

The reaction with rate constants is

$$E_{ox} + \text{glucose} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_{ox}G \xrightarrow{k_3} E_{red} + P_1$$

$$E_{red} + \text{oxygen} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E_{red}O_2 \xrightarrow{k_7} E_{ox} + H_2O_2$$

The velocity constant $\phi_o = \frac{1}{k_3} + \frac{1}{k_7}$, $\phi_1 = \frac{k_2 + k_3}{k_1 k_3}$, $\phi_2 = \frac{k_6 + k_7}{k_5 k_7}$

The value of these constants for the enzyme at pH10.0 is $\phi_o = 0.175 \times 10^{-4}$min, $\phi_1 = 3.93 \times 10^{-7}$ M.min, $\phi_2 = 8.04 \times 10^{-9}$ M.min. After photochemical reaction the constants were $\phi_o = 0.282 \times 10^{-4}$ min, $\phi_1 = 8.7 \times 10^{-7}$ M.min, $\phi_2 = 1.65 \times 10^{-8}$ M.min.

Fig.8.3. Change in enzyme spectrum with light under various conditions.

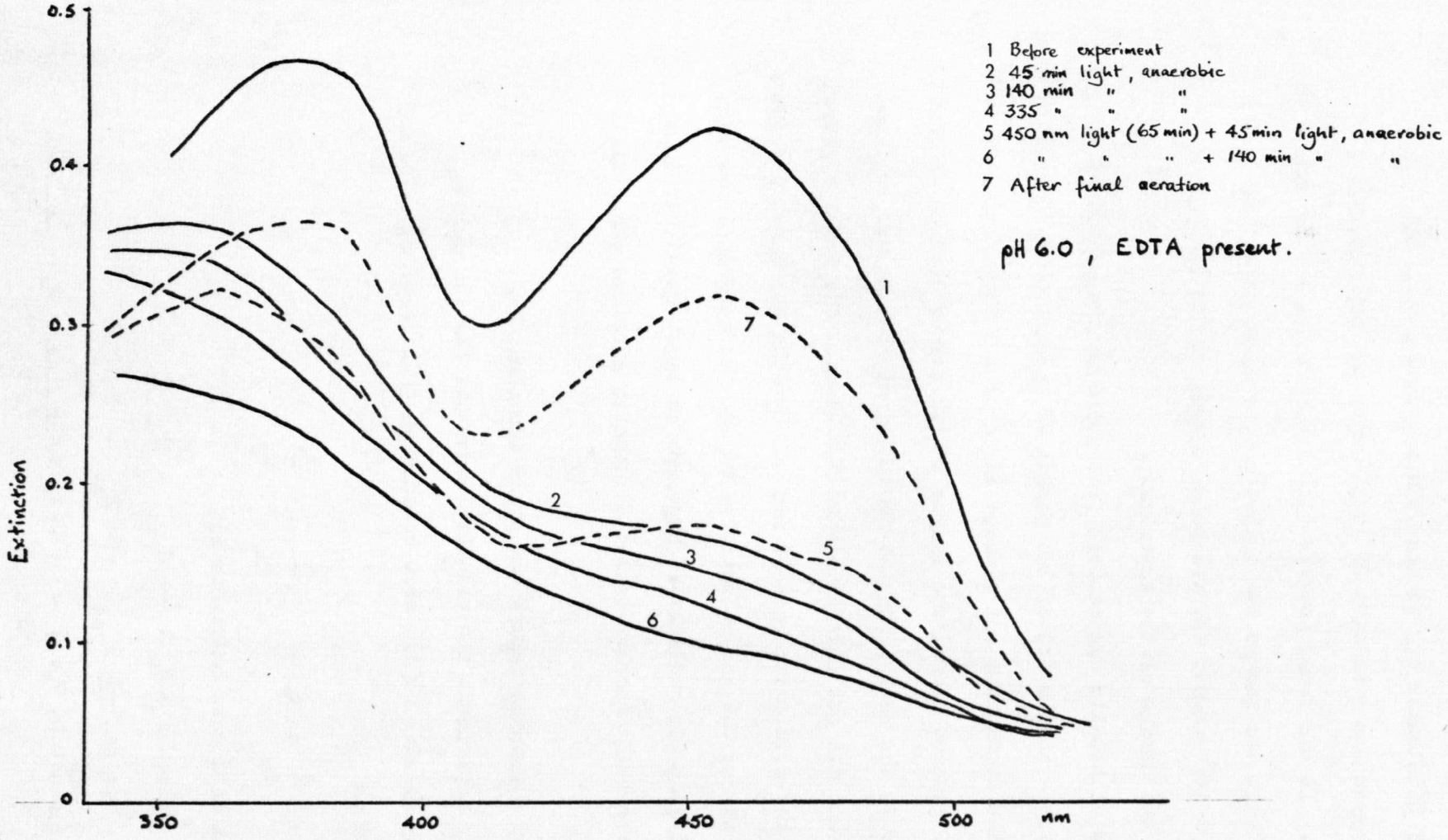
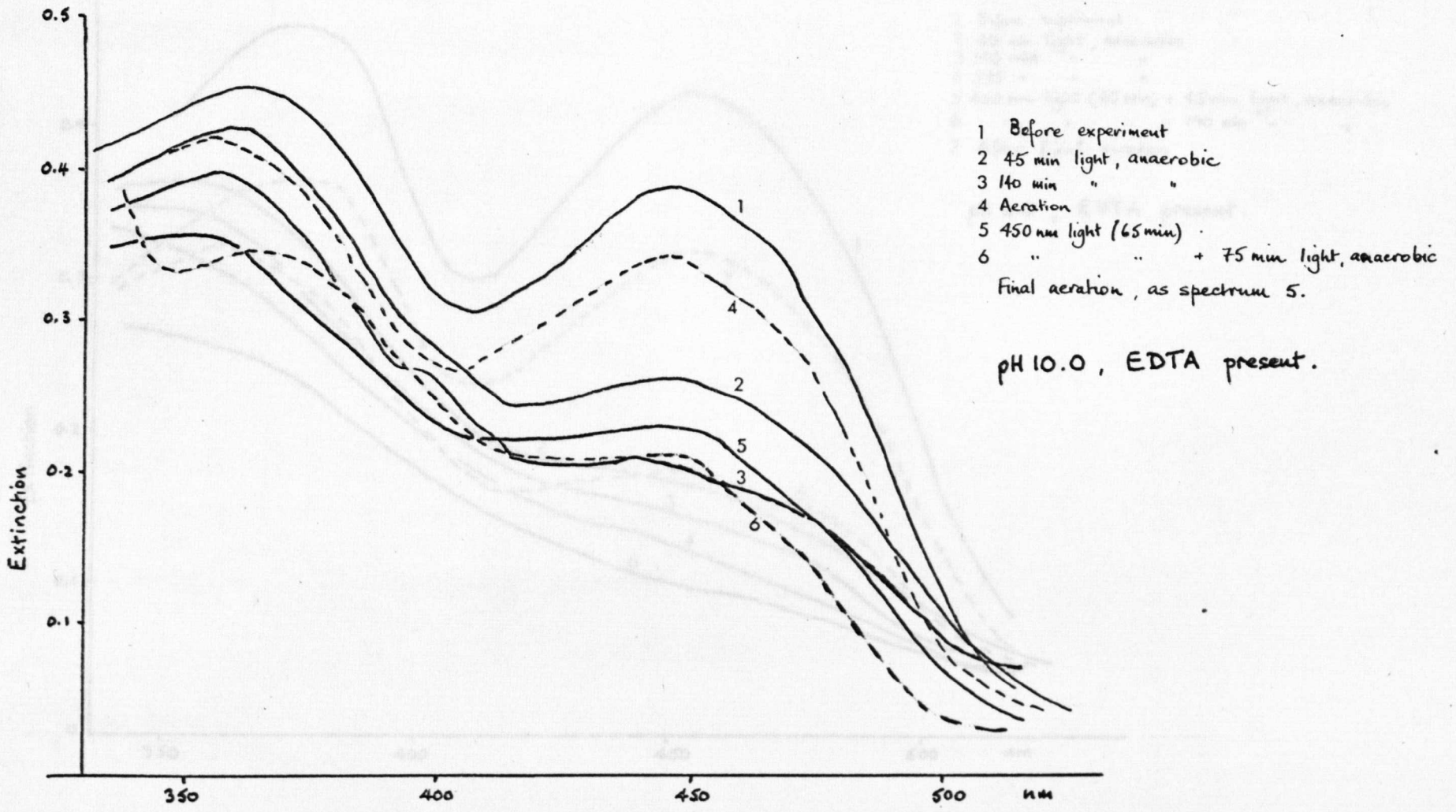


Fig.8.4. Changes in enzyme spectrum with light under various conditions.



7. Anaerobic titrations.

These experiments were modifications of the titrations described by Massey and Palmer (1966). Experiments were carried out at pH 6.0 and 10.0 as follows:

(1) The spectrum of the enzyme solution (containing 0.03 M-EDTA) was measured from 700 nm (adjusted to zero absorbance) to 300 nm.

(2) The sample was evacuated three times (see "Materials and Methods") and exposed to white light. The spectrum was measured at periods of time up to $5\frac{1}{2}$ hours.

(3) The sample was then restored to atmospheric pressure and the spectrum taken after 8 hours, in equilibrium with air.

(4) The sample was exposed to 450 nm light for 65 min.

(5) The sample was evacuated again and exposed to white light, the spectrum being measured at intervals as before.

The changes in the enzyme spectrum during the course of the experiment are shown in fig.8.3 and 4. At pH 6.0 the enzyme flavin spectrum was reduced by the photochemical reaction (with EDTA, under vacuum). A blue shift of the 380 nm peak was observed. When air was readmitted to the cell, the normal oxidised spectrum was obtained. The effect of monochromatic light at 450 nm was to reduce the spectrum. The subsequent photochemical reaction produced a blue shift in the spectrum, which again returned to the oxidised spectrum on admission of air at the end of the experiment.

At pH 10.0, the photochemical reaction caused the reduction of the flavin spectrum with a red shift. Admission of air to the cell restored the normal oxidised spectrum. After exposure to 450 nm light, the spectrum was reduced and the photochemical reaction which followed brought about a considerable red shift of the peak at 380 nm. When air was finally admitted to the cell, only a very slight change of the reduced spectrum was noted.

Discussion

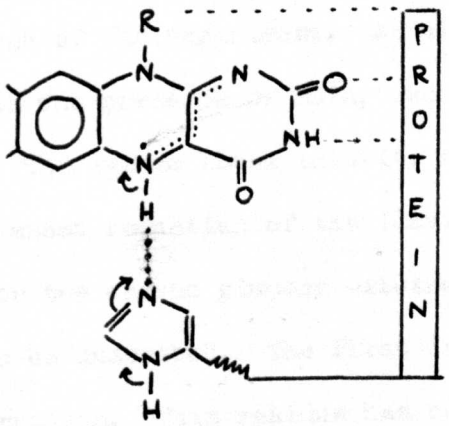
The photo-chemical reaction at 450 nm, pH 9.0 and 10.0 in the presence of EDTA has been shown to be insensitive to the presence of air, and therefore cannot be called photo-oxidation. The light in the presence of EDTA and at pH 10.0 brought about the inactivation of the enzyme in its oxidised state. Glucose protected the enzyme from this inactivation. This type of inactivation suggested that photo-reduction might be occurring (McCormick, Koster and Veeger 1967). Neither the addition of glucose to the inactivated enzyme, nor the dissolved oxygen in the assay solution restored activity. Experiments with dithionite indicated that light affected the enzyme in both its oxidised and reduced forms, and it is concluded that photo-destruction rather than reduction was occurring.

From the spectrum (fig.8.4) and from the graph of loss of activity with time (fig.8.1.) it can be seen that the enzyme was not fully destroyed. The remaining activity was sufficient to allow the velocity constants to be measured. The two-fold change obtained in ϕ_1 and ϕ_2 indicated that the enzyme unaffected by the light reaction differed only slightly in kinetic properties from the native enzyme. It is possible that a very small proportion of the enzyme sample had a different protein conformation to the rest and that this protected it from the photo-destruction. This would also explain the differences in velocity constants.

Hemmerich, Nagelschneider and Veeger (1970) have suggested that it is a histidine residue of the enzyme protein which stabilises the flavoprotein semiquinone produced on half-reduction of glucose oxidase in the absence of substrate. The enzyme exhibits both a red semiquinone anion above pH 7.5 and a blue semiquinone species below pH 7.5, when photo-chemically reduced in the presence of EDTA (Massey, Muller,

Feldberg et al 1969). The results in the previous chapter have indicated a group in the enzyme with a pK of 7.2, which was susceptible to photo-oxidation, which was assigned to a histidine residue. Experiment (7) was designed to test whether this histidine was the one which would also stabilise the semiquinone of the flavin generated by a photo-chemical reaction.

The scheme of Hemmerich et al (1970) is shown below.



For the enzyme sample at pH 6.0, aerobic exposure to light at 450 nm (in the presence of EDTA) brought about reduction of the flavin spectrum. This reduction was reversed at the end of the experiment when the enzyme sample was shaken with air. In the identical experiment at pH 10.0 the reduced spectrum was not reversed on shaking the enzyme with air. Thus if a histidine residue in the enzyme protein is being photo-oxidised at pH 6.0 it cannot be in close proximity to the FAD moiety, whose reduced spectrum changed to the oxidised form on exposure to air, or it would have affected the spectrum. Moreover the blue semiquinone of the enzyme flavin was obtained both before and after the photo-oxidation reaction. If the same histidine residue which, it is proposed, stabilises the semiquinone had been photo-oxidised

then no semiquinone should have been observed in the second part of the experiment (i.e. after exposure to light at 450 nm).

In the case of enzyme at pH 10.0 a red shift was observed during the photo-chemical reactions with EDTA, under vacuum. This indicated the presence of a red semiquinone form of the flavin. Again if a histidine residue was photo-oxidised during the exposure to light at 450 nm, then it could not have been the same one which stabilised the semiquinone. However after this treatment the enzyme remained in the fully reduced form and the spectrum was not oxidised on admission of air at the end of the experiment. As discussed above, at high pH, light at 450 nm, in the presence of EDTA, caused photo-destruction of part of the enzyme. The enzyme loses activity and as the spectrum shows, suffers permanent reduction of the flavin.

Thus for the enzyme glucose oxidase three types of photo-chemical reaction can be described. The first is photo-oxidation of a residue in the enzyme protein. This residue has been tentatively identified as a histidine residue. Photo-oxidation of this type occurs in the presence of a sensitising dye. The second type of photo-chemical reaction is that at high pH in the presence of EDTA and in the absence of oxygen. This was described as photo-reduction and admission of air restored the enzyme to the oxidised state. The third type of photo-chemical reaction was that at high pH, in the presence of EDTA using light of 450 nm. The enzyme lost activity which could not be restored by oxygen or glucose. This was photo-destruction, which was insensitive to air and it seemed to affect the enzyme in both the oxidised and reduced states.

CHAPTER NINE

DISCUSSION OF POSSIBLE MECHANISMS FOR GLUCOSE OXIDASE.

Apart from the initial investigations of P. amagasakiense by Kusai (1960) and Kusai et al (1960), there has been little work done with this enzyme species. One of the aims of the project was to follow up and extend the work of Kusai. In particular, it was hoped to investigate his surprising finding that the apo-enzyme would combine with FMN and that this FMN-enzyme could oxidise glucose with dichlorophenol-indophenol (DCIP) as the hydrogen acceptor but that it could not use oxygen as the hydrogen acceptor. Swoboda (1969b) has shown that in glucose oxidase of A. niger the FAD is probably bound by its adenine, ribose phosphate and dimethylisoalloxine residues. He found no regeneration of enzyme activity on adding FMN to the apoenzyme, either by itself or in combination with AMP (adenosine-5 phosphate). Kusai's finding would mean that the bond between the adenine of the FAD and the protein was not essential for the reconstitution of the enzyme. In the absence of the AMP moiety i.e. with FMN, the enzyme showed a different specificity for the hydrogen acceptor.

A change in enzyme activity on binding FAD to the apo-enzyme has been reported for lipoamide dehydrogenase (Kaise and Veeger 1968). In this case on reconstitution of the enzyme at 0-5°C, the enzyme activity towards lipoate was low while the activity towards the artificial electron acceptor, DCIP, was high. As the temperature was raised so the lipoate activity increased and the DCIP activity decreased. It had been hoped to follow up the findings of Kusai, in the hope of finding a molecular explanation for the differences in properties of the FAD and FMN enzymes. However Kusai's preparation of the apo-enzyme could not be repeated.

In chapter 4 on cation inhibition, it was assumed that Ag^{+}, Hg^{2+} and Cu^{2+} ions were all reversible inhibitors at the same site. The silver ions bound much more strongly than the otherions. Nakamura and Ogura (1968) investigated the binding of Hg^{2+} and Ag^{+} ions to A. niger glucose oxidase. They showed that the two ions combine at different sites on the enzyme. They also found a difference in the shape of the reciprocal plots for the ions. For the silver ion, the plots showed that it competed with oxygen for the reduced enzyme. In the case of mercury, neither competitive nor non-competitive inhibition was obtained with respect to glucose. Mercury was thought to combine with the oxidised enzyme, the enzyme-glucose complex and the reduced enzyme. Subsequent kinetic analysis showed that mercury competitively inhibits the enzyme with respect to oxygen. Nakamura and Ogura have suggested that Hg^{2+} ions bind strongly to the reduced enzyme but weakly to the acid enzyme.

The reaction of P. amagasakiense glucose oxidase with bisulphite was investigated and reported in chapter 5. The results obtained from kinetic and spectral experiments showed that the bisulphite competed with glucose for the oxidised enzyme, which was bleached in a reaction which was clearly different from reduction. The combination of enzyme and bisulphite was found to be dependent on the concentration of the reactants and on the pH, which was also shown by Swoboda and Massey (1966). The most striking properties of the bisulphite adduct are (i) its spectrum, which is similar to that of the reduced flavoprotein, (ii) it will not react with oxygen, (iii) it is formed reversibly in a stoichiometric reaction in which one mole of sulphite is bound per mole of enzyme flavin. Swoboda and Massey(1966) showed that the reaction of bisulphite with their enzyme brought about shifts and large increases in the blue fluorescence (emission at 450 nm,

excitation at 360 and 290 nm), and in the ultra-violet fluorescence (emission at 340 nm, excitation at 290 nm). This latter fluorescence is a property of the aromatic amino-acids in the protein, and was used to follow the bisulphite binding to P. amagasakiense enzyme. An increase in the ultra-violet fluorescence was found (using excitation at 295 nm, emission at 350 nm) but it decayed over the following five minutes. The decay followed a first order reaction rate, with $k = 0.018\ min^{-1}$, and was independent of bisulphite concentration. This may have been due to contaminating metal ions catalysing the oxidation of the bisulphite, since no EDTA was present. In the comparable spectral experiments, in which EDTA was present, no slow reversal of bleaching was found.

In experiments on the inhibition of enzyme activity by bisulphite at several pH values, the inhibition constant of the initial reaction K, was obtained. From comparable spectral studies K, the equilibrium constant between enzyme and bisulphite was calculated. There was a measure of agreement in K from the two sets of experiments and Dixon plots of $-\log_{10}K$ against pH were drawn. From these it was found that there was an ionisable group in the enzyme which had a pK of 4.2. As discussed in chapters 5 and 6, the most likely group with such a pK is the amino group of the adenine of FAD. Experiments on the binding of halide anions to the enzyme indicated an ionisable group with the same pK being involved. Swoboda and Massey (1966) studied the binding of bisulphite to the glucose oxidase from A. niger. Using the Dixon plot, ionisable groups with pKs of 4.75 and 6.55 were implicated in the binding. From the shape of the plot, in accordance with the Dixon rules (Dixon 1953) they ascribed the pK of 4.75 to an ionisable group in the enzyme which had to be protonated for bisulphite binding to occur. The pK of 6.55 was sufficiently close to the second pK of sulphurous acid (6.91) to be assigned to the equilibrium between the

bisulphite and sulphite anions.

In a recent paper on "The glucose oxidase mechanism" Weibel and Bright (1971) using A. niger enzyme have examined the effect of halide ions on the enzyme reaction at pH values between 3 and 10. At low pH they found that the presence of halide ions shifted the apparent pK_1 of 2.5 - 3.0 to a higher value. For P. notatum enzyme, Bright and Appleby (1969) found a shift in the pK_1 from 4.0 to 5.0 in the presence of halide. On the strength of the value of the low apparent pK_1, in the A. niger enzyme, Weibel and Bright have assigned to it the ionisation of a carboxylate group in the protein. Experiments on the maximum turn-over number (k_{cat}) in the presence of halide ions at low pH have shown it to be entirely determined by the rate of flavin reduction (k_2) in the reductive half-reaction, below,

$$E_{ox} + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_{ox}S \xrightarrow{k_2} E_{red} + \delta\text{-lactone} \quad \ldots\ldots\ldots (1)$$

In the absence of halide ions k_{red} decreased at low pH. Assuming rapid equilibrium, $k_{red} = (k_1 k_2)/(k_{-1})$. As a result of these findings Weibel and Bright suggested that at low pH the enzyme exists in a protonated and an unprotonated form.

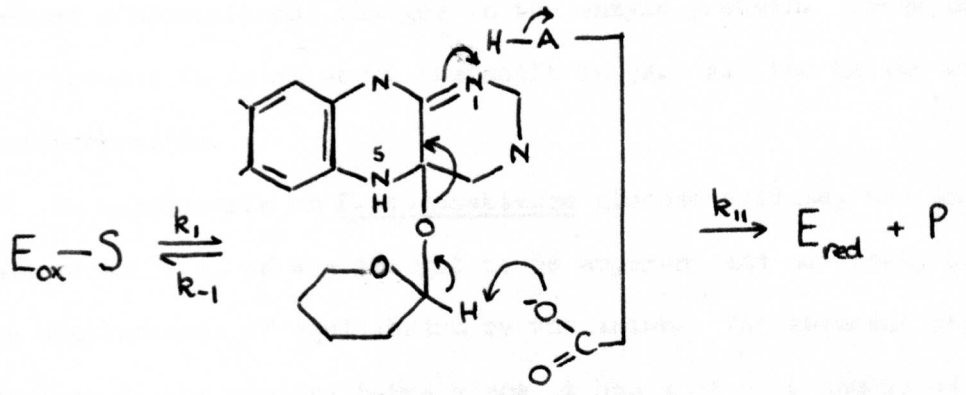
$$E_{ox} + H^+ \rightleftharpoons E_{ox}H^+ \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

Such a conclusion had been drawn for P. notatum enzyme also (Bright and Appleby 1969).

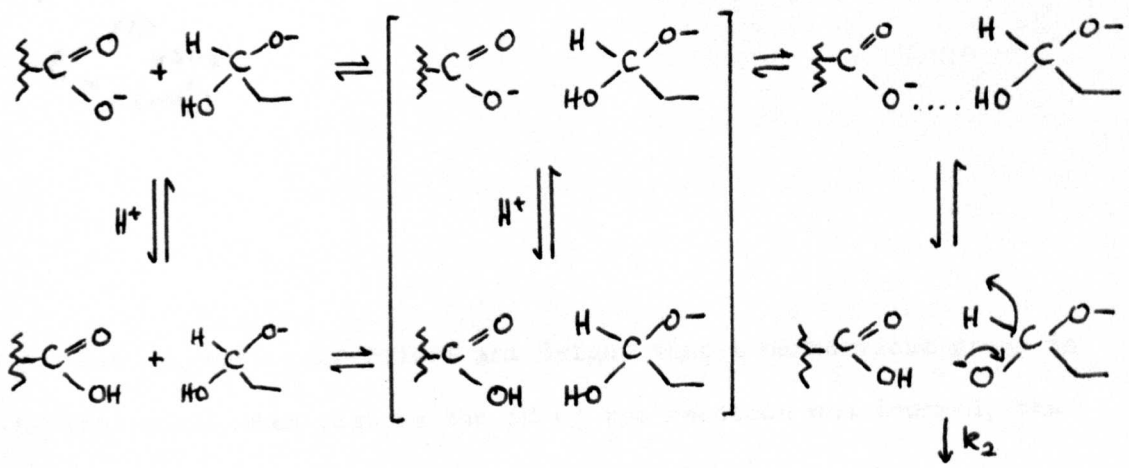
Weibel and Bright (1971) have suggested two theories for the mechanism of oxidation of the glucose substrate by the heterocyclic nucleus of FAD. Both mechanisms could be assisted by general base catalysis.

(1) Nucleophilic attack on the flavin nucleus by the glucose C-1 hydroxyl group followed by proton removal from the carbon and electronic rearrangement. This flavin-adduct hypothesis shown below requires a group H-A which could be H_2O or an acidic enzyme residue

with pK_a greater than 10.



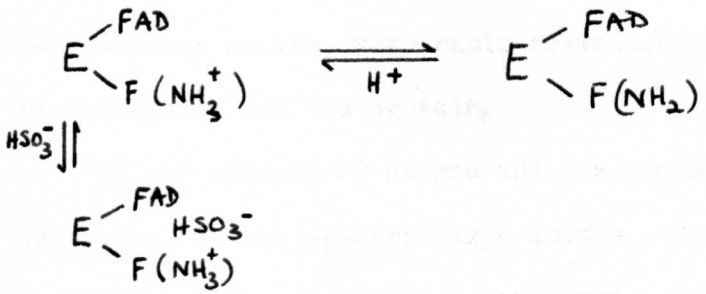
(2) Hydride transfer from the glucose carbon to the flavin, shown below.



In mechanism (1) a carboxylate group assists in proton removal after the attack by the glucose on the flavin. In mechanism (2) that carboxylate anion plays an important part both in binding glucose and in hydride transfer, which is assumed to proceed to the electron sink of the flavin. Weibel and Bright, while favouring mechanism (2) could not rule out mechanism (1) on the basis of their experimental results. They postulated the involvement of a carboxylate anion on the basis of

their apparent pK_1 value of 2.5 - 3.0, in the absence of halide ions. The inhibitory effect of halide ions at low pH was attributed to halide induced conformational changes in the enzyme protein. These changes were thought to bring about the shift in pK. All the halide effects were reversible.

In experiments on P. amagasakiense glucose oxidase, the halide induced shift in pK was thought to be apparent and not real, because of the displacement of equilibrium by the anion. The apparent pK_1 was ascribed to the adenine 6-NH_2 since it has a pK of 4 and is adjacent to the flavin. Thus the binding of negatively charged ions could be pictured as:-



The suggestion of Weibel and Bright that a carboxylate group is involved would mean that as the pH of the reaction was lowered, the group would become protonated (equation 2 above),

$$E\text{-}COO^- + H^+ \rightleftharpoons E\text{-}COOH .$$

Under such circumstances, it might be expected that anions like bisulphite and halide would not bind to either the protonated or unprotonated enzyme. Experimental data showed that binding of anions increased as the pH of the reaction was lowered, and this provided circumstantial evidence for the involvement of the adenine 6-NH_2 group.

The photo-oxidation experiments indicated that a group with pK of 7.2, probably a histidine residue, was involved in the glucose oxidase

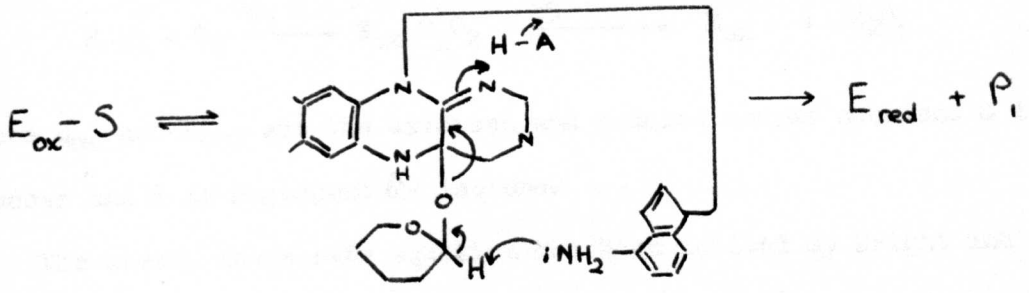
reaction. Whether it was involved in the reductive or oxidative part of the reaction was not investigated.

In their investigation of the reaction of reduced enzyme with oxygen, Weibel and Bright have found an apparent pK of 7.5. While it is close to the value of pK 6 - 7 for the N-1 hydrogen of the reduced isoalloxazine ring, they prefer another explanation. This is that the apparent pK is derived from an intramolecular equilibrium process coupled to proton transfer, which disguises the true pK. This derives from the dissociation of the N-3 proton from the reduced flavin, with subsequent attainment of a low-energy structure of reduced isoalloxazine. After oxidation they propose that re-protonation of the N-3 position occurs, with rapid formation of the hydrogen bond between this position and the protein.

In the absence of oxygen and dye another type of photo-reaction occurred. In the spectral experiments of chapter 8 a definite reduction of the spectrum was obtained. The source of electrons in these experiments was EDTA. This photo-reduction was most marked at pH 10.0, while at pH 6.0 no permanent reduction of the spectrum of the enzyme was obtained. At pH 10.0 a red shift in the enzyme spectrum was noted during photo-reduction, indicating the possible formation of a red semiquinone. At pH 6.0 a blue shift was obtained during the photochemical reaction indicating blue semiquinone formation. Hemmerich, Nagelschneider and Veeger (1970) suggested that the semiquinone of glucose oxidase might be stabilised by a histidine residue in the protein, (see chapter 8). The above experiment may be interpreted as showing that the histidine which may have been photo-oxidised by 450 nm light was not the same histidine proposed by Hemmerich et al. At pH 10.0 the photo-chemical reaction brought about permanent reduction of the enzyme (flavin) spectrum, and

irreversible loss of activity i.e. photo-destruction of a vital residue in the protein for catalytic activity.

Recent studies (Brown and Hamilton 1970) on model compounds have shown that the likely site of substrate attachment and attack on the flavin is at position C-4a. In the light of these and other experiments a speculative mechanism for the reduction of the flavin moiety of glucose oxidase is outlined below.



Glucose oxidase is a 2 substrate enzyme, having a double displacement mechanism. The minimal mechanism for this reaction has been proposed by Gibson, Swoboda and Massey (1964) and Bright and Gibson(1967) from studies using glucose, 2- deoxy glucose and 1-deutero glucose,

$$E_{ox} + G \underset{k-1}{\overset{k+1}{\rightleftharpoons}} E_{ox}G \xrightarrow{k2} E_{red}\,P \xrightarrow{k3} E_{red} + P$$

$$E_{red} + O_2 \xrightarrow{k4} E_{ox}\,H_2O_2 \xrightarrow{k5} E_{ox} + H_2O_2$$

where E_{ox} and E_{red} are the oxidised and reduced enzyme species, G is glucose and P is D-glucono δ- lactone.

The steady state rate equation has been derived by Bright and Gibson (1967) and in Dalziel notation (Dalziel 1957) such a double displacement mechanism may be written as

$$\frac{e}{v_o} = \phi_o + \frac{\phi_1}{S_1} + \frac{\phi_2}{S_2}$$

where e is initial enzyme concentration, v_o is the initial velocity of the reaction, ϕ_o , ϕ_1 , ϕ_2 are constants which can be determined from related groups of velocity constants, S_1 and S_2 are the concentrations of glucose and oxygen respectively.

An inhibitor could combine with any of the enzyme or enzyme-substrate species in the glucose oxidase reaction mechanism. To ascertain the nature of the inhibition and whether there is competition between inhibitor and substrates for any enzyme species double reciprocal plots are drawn. That is, the reciprocal initial velocity, with and without inhibitor present, is plotted against the reciprocal of glucose concentration at constant oxygen concentration. Then the same process is carried out but the reciprocal oxygen concentration is plotted, with

glucose concentration constant. From the shape of the resulting graphs the inhibition pattern can be predicted. This treatment and the significance of the resulting linear plots has been described by Lineweaver and Burk (1934) Alberty (1956) and Dalziel (1957), and the interpretation for a one substrate enzyme reaction is summarised below.

When the lines for inhibitor present and absent intersect on the reciprocal velocity axis, it is a case of competitive inhibition where the inhibitor reacts only with the native enzyme.

When the lines only intersect on the reciprocal substrate axis, it is a case of non-competitive inhibition where the inhibitor reacts with both native and enzyme-substrate complex.

When the lines are parallel, crossing the reciprocal velocity axis it is a case of uncompetitive inhibition where the inhibitor combines with the enzyme-substrate complex only. For the two substrate mechanism above, if the inhibitor combines with E_{ox}, competitive inhibition is shown on the plot of reciprocal velocity against reciprocal glucose. If inhibitor combines with E_{red}, competitive inhibition appears on the plot of reciprocal velocity against reciprocal oxygen.

From the reciprocal plots, the inhibition constant, K_I, can be obtained as follows.

With no inhibitor present

$$\frac{e}{v_o} = \phi_o + \frac{\phi_1}{S_1} + \frac{\phi_2}{S}$$

The reciprocal plot of S_1 (with S_2 held constant) gives a line whose slope is ϕ_1

With inhibitor present and competing with S_1 for the enzyme

$$\frac{e}{v_o} = \phi_o + \frac{\phi_1}{S_1}\left(1 + \frac{I}{K_I}\right) + \frac{\phi_2}{S_2}$$

The same reciprocal plot of S_1 now has a line whose slope is

$$\phi_1 \left(1 + \frac{I}{K_I}\right) = \phi_1'$$ where I is inhibitor concentration .

The ratio of the slopes is

$$\frac{\phi_1'}{\phi_1} = \left(1 + \frac{I}{K_I}\right)$$

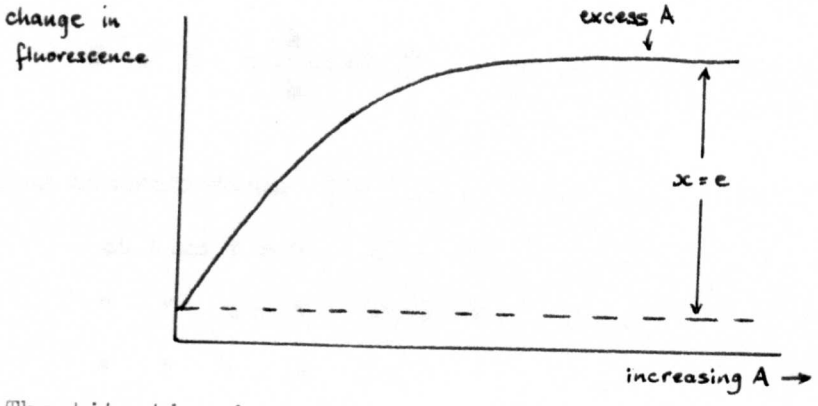
$$K_I = I \left(\frac{\phi_1}{\phi_1' - \phi_1}\right)$$

Experimental method.

The pen recorder attached to the oxygen electrode was calibrated at two points with respect to the buffer solution (25°C). With buffer solution in the cell, the signal from the electrode was set at 100% oxygen concentration on the recorder scale. The oxygen saturation of the buffer solution was taken to be 2.6×10^{-4} M, the same as that of water in equilibrium with air at 25°C and at atmospheric pressure. Then dithionite was added to the cell to reduce the molecular oxygen in the solution. This was followed on the recorder and when no further change in the electrode signal was obtained, the recorder scale was set to 0% oxygen concentration. No other calibrations eg. with solutions of known oxygen tension, were carried out. It was assumed that the recorder responded linearly to change in oxygen tension of the cell contents.

Recordings of the change in oxygen concentration of the experimental solution with time were made at several glucose concentrations, usually 6. For each glucose concentration the velocity of the reaction was obtained at 100% oxygen concentration by taking the tangent to the curve at that point on the recorder scale. For a fixed glucose concentration, the reaction rate at various oxygen concentrations was found by taking tangents to the curve at several points shown by the recorder as 90%, 80% 70% etc. (All buffer and sugar solutions had air bubbled through them for 10 minutes before use.)

The effect of the titration may be followed in many ways; in this example the change in fluorescence of a protein is used.



The titration is described by the following equation

$$P + A \rightleftharpoons PA$$

In terms of concentration $(e - x)(a - x) \rightleftharpoons x$

where P is the protein, A the monovalent ligand, e, x and a are the concentrations of protein, protein-ligand complex and ligand respectively.

$$f = \frac{\text{fraction of sites occupied by ligand}}{\text{total number of binding sites}}$$

$$= \frac{x}{e}$$

By definition the dissociation constant

$$K = \frac{(e-x)(a-x)}{x}$$

Substituting for $x = ef$

$$K = \frac{(e-ef)(a-ef)}{ef} = \frac{(1-f)(a-ef)}{f}$$

$$\frac{a}{f} = \frac{K}{(1-f)} + e$$

a/f is plotted against $1/(1-f)$ giving a line whose slope is K and intercept e

In the case of bisulphite titrations which were followed by the change in optical density at 450 nm, a series of experiments were required to give

a range of values of enzyme-bisulphite complex formation. Finally the OD_{450} was measured when the bisulphite concentration was infinitely great, giving the value of maximum change

$$E + B \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EB$$

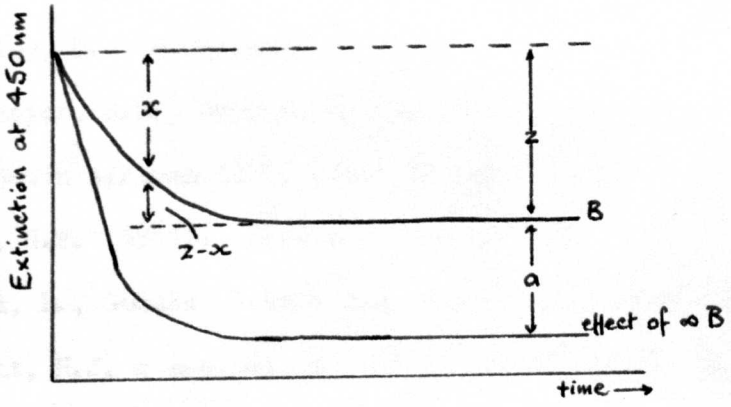
where E is enzyme, B bisulphite
EB is enzyme-bisulphite complex

Taking concentrations for E , B and EB in the above equation

at time t = 0, a + B = 0

" " t = t, a-x + B = x

" " t = ∞, a-z + B = z , at equilibrium



The kinetic condition is $\frac{dx}{dt} = k_1 (a-x)B - k_2x$

The equilibrium condition is $\frac{dx}{dt} = k_1(a-z) B - k_2z$

By integration

$$\log_e \frac{z}{(z-x)} = (Bk_1 + k_2)t$$

Plotting $\log_{10} \frac{z}{(z-x)}$ against t, the gradient of the line

at any one bisulphite concentration is $(Bk_1 + k_2)$ /2.303 and intercept $\log_{10} z$

From 2 different bisulphite concentrations, k_1 can be determined by subtraction of the slopes, since k_1 varies with bisulphite concentration.

Then K_I (inhibition constant) = $\frac{k_2}{k_1}$

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Proteolytic enzymes from extremely halophilic bacteria

H.Geisow.

This supplement is presented in part-fulfilment of the degree of Doctor of Philosophy, University of Warwick. It represents the work carried out under the supervision of Dr.J.Stevenson, in the School of Molecular Sciences, University of Warwick, from October 1967 to December 1968.

Summary.

Both extracellular and intracellular proteases have been demonstrated for Halobacterium salinarium, an extremely halophilic bacterium. The extracellular enzyme is active in the presence of high concentrations of sodium chloride.

Some proteolytic activity was associated also with the cell membranes, and with the soluble cell contents released by sonication of the cells. Both proteases were inhibited by high sodium chloride concentrations, and may have been the same enzyme. A pH optimum of 7 was obtained for intracellular proteolytic activity.

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Abbreviations.

Recommended S.I. symbols for units have been used. In this and other respects the "Instructions to Authors" of the Biochemical Journal, published by The Biochemical Society, London, 1971, have been followed.

EDTA	ethylenediaminetetraacetate
tris	tris (hydroxymethyl) aminomethane

Introduction.

The reddening of salted protein products (fish, hides, etc.) was recognised as being of microbial origin around the turn of the century. The general bacteriology of these salt-loving microbes was investigated by Klebahn (1919) and Harrison and Kennedy (1922), who showed them to be highly specialised organisms.

Some micro-organisms are capable of reproduction and growth in a medium of high salt content (e.g. see Prescott and Willms, 1960). Some are obligate halophilés, capable of growth only in that medium. In this latter group are the "extreme halophiles", bacteria that absolutely require strong, almost saturated, brine for growth. Larsen (1962) in a review of the biochemistry of the extreme halophiles, showed that they belonged to one of two distinct groups:

(1) the Halobacterium group,

(2) the Sarcina-Micrococcus group

Both groups of bacteria are characteristically red, pink or orange in colour owing to the presence of carotenoids. The extreme halophiles are usually found in strong saline solutions under bright sun (e.g. the salt pans), and it has been suggested that the caroteniods protect the organism from the sun's deleterious effects. Comparative experiments with an artificially produced colourless mutant have demonstrated the protective role of the carotenoids (Dundas and Larsen 1962, 1963).

The Halobacteria are distinguished from the Sarcina group by more extreme halophilism. They require an optimal salt concentration of 25 - 30% (w/v) compared with 20-25% (w/v) for the latter group. Dilution of the saline environment causes drastic morphological changes in Halobacteria, leading to cell lysis at 5-10% (w/v) salt.

Extreme halophiles show a specific salt requirement. Mohr and

Larsen (1963) using H. salinarium, have made a comprehensive study of the effects of substituting other salts for NaCl. They found that only sodium acetate, potassium acetate and potassium chloride would preserve the morphology of the bacteria. Some salts e.g. sodium iodide and cadium chloride, would protect the organism against lysis though not against changes of shape. Experiments to get growth in media containing salts substituted for NaCl have failed so far (Klebahn 1919, Hess 1942, Weber 1949).

Baxter and Gibbons (1954, 1956, 1957) first discovered that the enzymes of the extreme halophiles are adapted to function at the very high salt concentrations found within the cells. They used crude extracts of H. salinarium and tested the response of several enzymes in the extracts towards various different concentrations of NaCl. In all cases there was good activity at salt concentrations corresponding to those found in the cells under normal culture conditions. Most of the enzymes showed little or no activity at low salt concentrations. The range of enzymes studied has been extended by Holmes, Dundas and Halvorson (1965). Their results support the hypothesis that enzymes of extreme halophiles are extremely halotolerant. Comparative experiments with Sarcina morrhuae (Larsen 1962) have shown that although the two groups of bacteria have different responses to salt, enzymes of the same type from the different bacteria display similar salt responses.

Baxter and Gibbons (1954, 1956, 1957) tested the effect of a number of salts other than sodium chloride, on the enzymes of H. salinarium. Several of the alkaline cations and the anions bromide and nitrate could replace Na^+ and Cl^- respectively. Of the anions K^+ was most potent in activating the enzymes. With potassium chloride, double the activity was obtained compared with that obtained with sodium chloride at the same molar concentrations. The strong stimulatory effect of both sodium and

potassium chlorides is consistent with the high intracellular concentrations of K^+, Na^+ and Cl^-. The data for the intracellular concentration of these ions (published by Christian and Waltho 1962) is given in table 1.

Table 1. Concentration of intracellular constituents of H. salinarium.

	molal equivalents
Na^+	1.37 ± 0.21
K^+	4.57 ± 0.12
Cl^-	3.61 ± 0.07

The molal concentration ratio of cell Na^+ to medium Na^+ was found to be 0.03, while the ratio of cell K^+ to medium K^+ was 143; for Cl^- the ratio was 0.80.

That the salts also act as stabilisers of the enzymes has been shown by dialysis experiments. In these, the salt had been dialysed away from the enzymes, resulting in denaturation (Baxter and Gibbons 1954-1957). Some enzymes regained activity if then dialysed against 25% (w/v) NaCl, but not on addition of solid NaCl (Holmes and Halvorson 1963, 1965).

The role of halophilic bacteria as food-spoilage organisms was highlighted by the report in the Times (June 23, 1966) of a consignment of salted wet fish, carried in an unrefrigerated ship, being damaged by these bacteria. If the bacteria were using the fish for food material then the bacteria were probably producing proteases extracellularly for protein digestion. Gibbons (1957) has tested 49 strains of extreme halophiles, and found that 45 of them possessed gelatinolytic activity. Most strains also degraded casein. Shah and de Sa (1965) reviewed the proteolytic activity of halotolerant and halophilic bacteria towards

gelatin-salt agar, milk-salt agar and fish muscle protein salt agar. H. halobium was found not to grow on the latter medium.

In 1969, Norberg and Hofsten published a paper on "Proteolyic enzymes from extremely halophilic bacteria". Using H. salinarium 1, a colourless mutant H. salinarium 1 M, and a vacuolated strain H. salinarium 5, they showed that a true extracellular protease was formed but that proteolytic activity depended on the strain used. In particular H. salinarium 1 showed low extracellular protease activity.

A particle-bound protease and a peptidase were identified in the cell homogenate. All the enzymes were sensitive to dialysis against EDTA in 25% (w/v) NaCl solution. Only the particle-bound protease was reactivated by Zn^{2+} or Mn^{2+} ions. All the enzymes rapidly and irreversibly lost activity in buffers which did not contain high concentrations of NaCl or KCl. Unlike the enzymes investigated by Holmes and Halvorson (1963, 1965) these proteases were not reactivated by dialysis against 25% NaCl.

Materials and Methods.

Bacterial strain. Halobacterium salinarium 1, originally obtained from Professor Helge Larsen, Trondheim University, was used.

Growth conditions. The bacteria were grown in Ian Dundas medium (IDM) containing 1% peptone (w/v): 250g NaCl, 5g NH_4Cl, 5g KCl, 5g $MgCl_2$, 10g peptone (Oxoid), tap water to 1 litre.

The bacterial cultures were started in Erlenmeyer flasks, using 5ml inoculum in 50 ml IDM per flask. The flasks were put on a rotary shaker for 3 days, at 37°C. Two Erlenmeyer flasks of bacteria were used as a starter for 500 ml IDM in a 1 litre flask, spun for 3 days on an orbital shaker, at 37°.

Measurements of growth. Cell densities of samples from liquid cultures

were measured in tubes of 16 mm diameter in an EEL colorimeter, using a red filter.

Determination of proteolytic enzyme activity. The method of Anson (1938) was used in a modified form, as detailed below. 1 ml enzyme solution (usually containing 25% (w/v) NaCl) was incubated with 1 ml 1% (w/v) solution of casein (Sigma) containing 0.005 M-$CaCl_2$ in 0.1 M-borate or tris buffer pH 8.0. The reaction was carried out at 30° and terminated after a period of time, by addition of 3 ml 5% trichloroacetic acid. After filtration, the extinction of the filtrate was read at 280 nm against a reaction blank for each sample, using a Zeiss PMQ II spectrophotometer. Suitable controls were carried out. The extinction at 280 nm was used as an indication of the presence of small peptides containing tyrosine or tryptophan, or of the amino-acids themselves.

Centrifugation. Separation of the bacteria from the medium was achieved by centrifugation at 7000 rpm for 30 min (Sorvall Inc.,) at room temperature.

Sonication. 2 ml samples of bacterial suspension were sonicated in an M.S.E. sonicator for 2 min at intervals of 30 seconds with 30 seconds rest, the samples being held at 4°C.

Results.

Studies on extracellular enzyme formation.

After 3 days growth, the bacteria were removed from the medium by centrifugation. The medium was dialysed against distilled water at 4° overnight. The nondiffusable residue was reduced to dryness, and taken up in 2 ml 2% NaCl solution containing 0.01 M-tris buffer pH 8.0. This was applied to a column (1.5 x 50 cm) of Sephadex-G50, fine grade (Pharmacia, Sweden), which had been pre-equilibrated with the NaCl-tris solution. The column was eluted using this solution (at 4°) and 10 ml

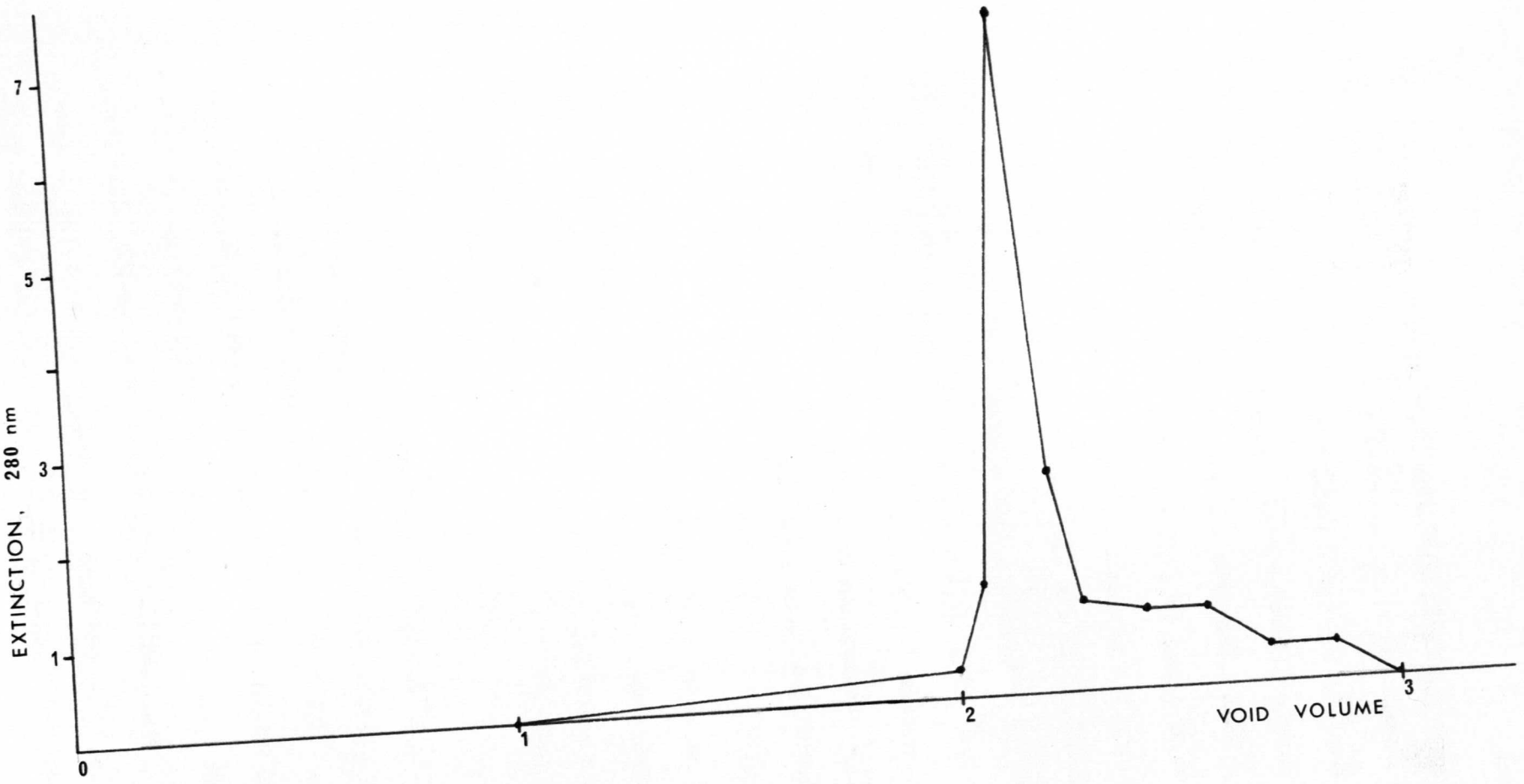

Fig.1. Difference in extinction of the medium before and after growth of H.salinarium.

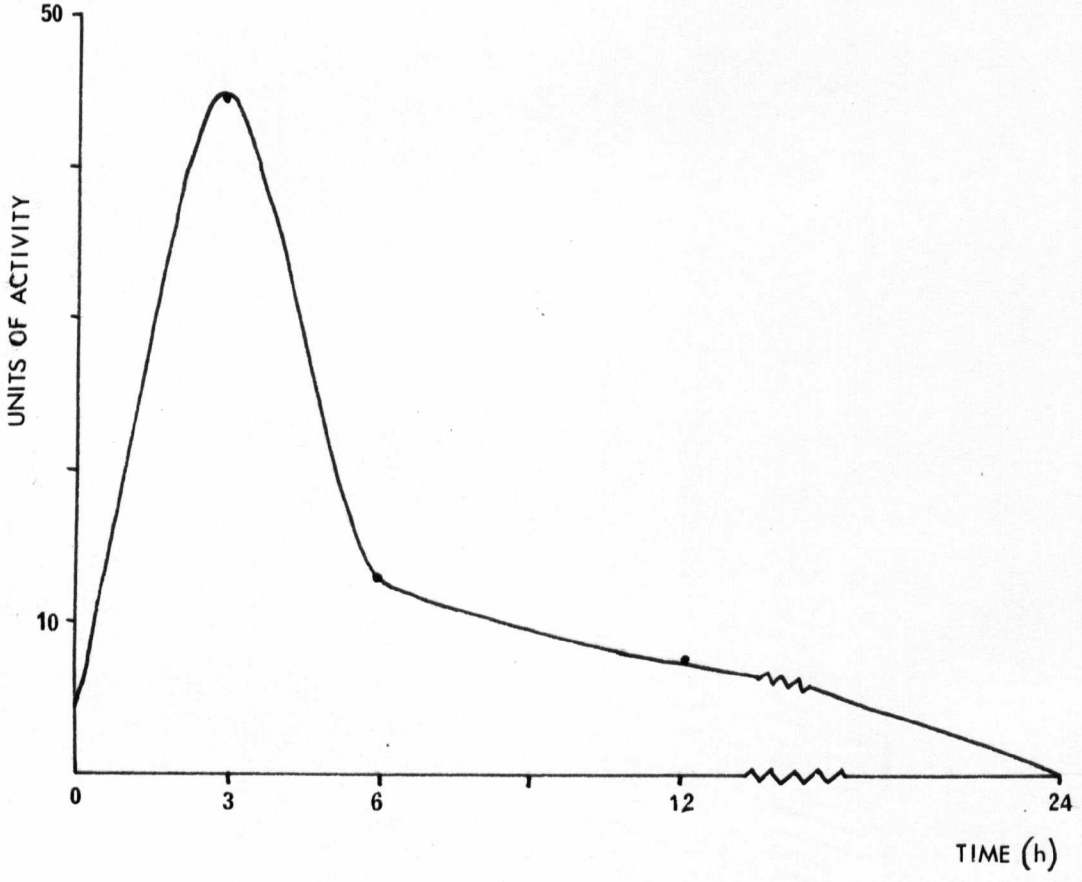

Fig.2. The presence of extracellular activity in the supernatant.

Bacteria were separated from growth medium, washed and resuspended in IDM and 0.5% glutamate. Samples of the suspending medium were removed at 3 hourly intervals and assayed for proteolytic activity, as described in the experimental section of the text.

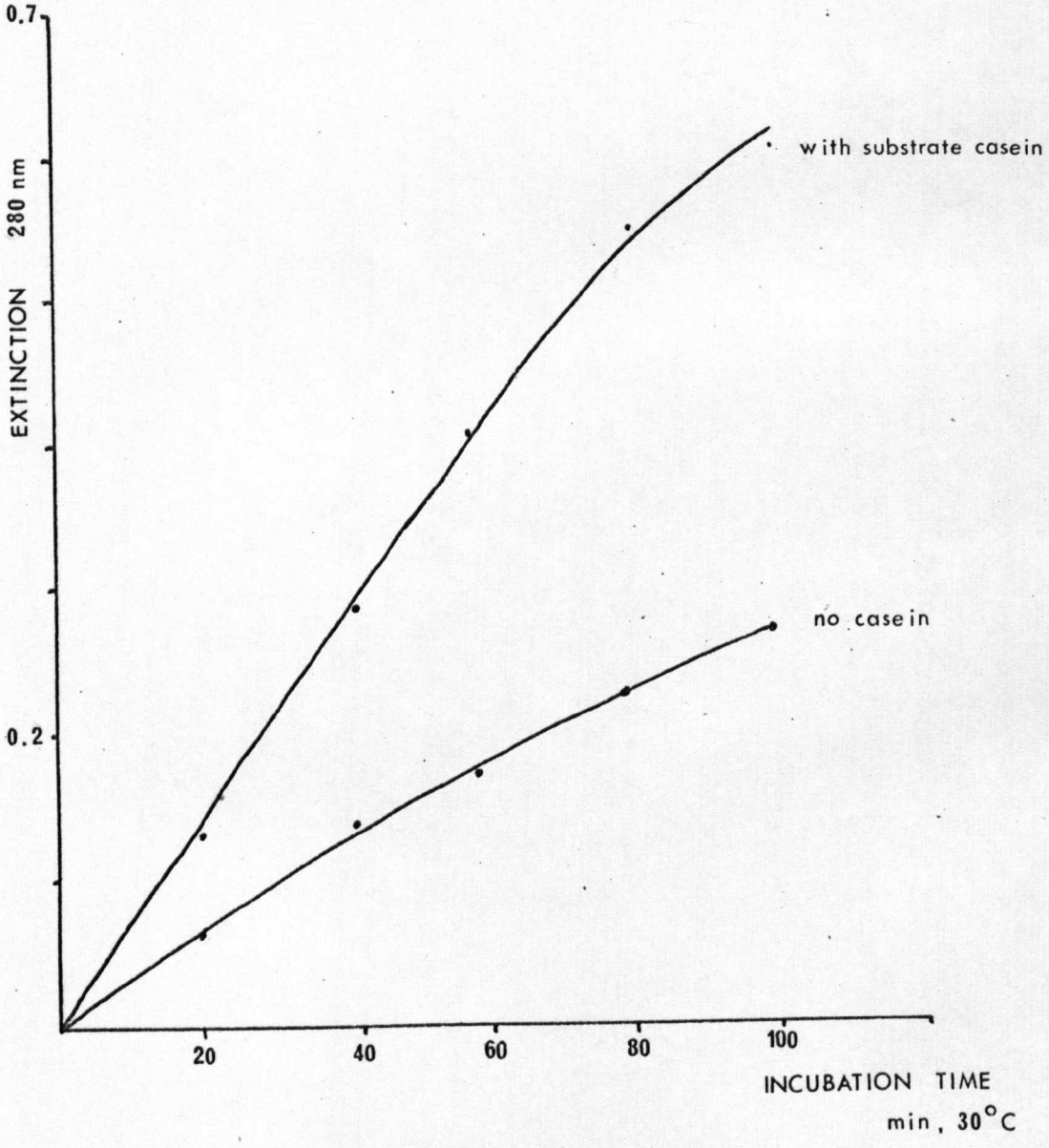

Fig.3. Activity of supernatant from sonicated cells.

Bacteria were separated from growth medium after 3 days, washed with IDM and sonicated to produce macerated cells. This was assayed for proteolytic activity as described in the text.

fractions were collected. The procedure was also applied to the medium alone, which had not been used for bacterial growth. The extinction of the column fractions at 280 nm was measured, as an indication of the presence of extracellular protein in the fractions. The difference in extinction between the two samples of media is shown in fig.1. Extracellular protein material was obtained in one peak, at about 2 void volumes.

The bacteria from the centrifugation above were washed in IDM, and resuspended in IDM + 0.5% sodium glutamate. Samples of the suspension were removed at 0,3,6, and 12 h, centrifuged and the supernatant was tested for proteolytic activity against casein,(fig.2.) Increased proteolytic activity was shown by the supernatant with time. The slight initial activity may have been due to cell breakage during centrifugation. Some bacteria were separated from the medium after 3 days growth, washed with IDM, and sonicated to produce macerated cells. This material showed considerable proteolytic activity, shown in fig.3. No activity was obtained from samples incubated at 0^{o}.

The proteolytic activity of supernatant from sonicated cells.

Harvested cells were resuspended in IDM/glutamate solution. Samples were sonicated and the material pooled. It was centrifuged (6,500 rpm, 8 min) and the precipitate was discarded. The supernatant was used for the following experiments, in which the high NaCl concentration was not maintained.

The activity of the supernatant was tested at several pH values. The enzyme solution was incubated for 40 min and the NaCl concentration was 5%. The results are shown in fig.4.

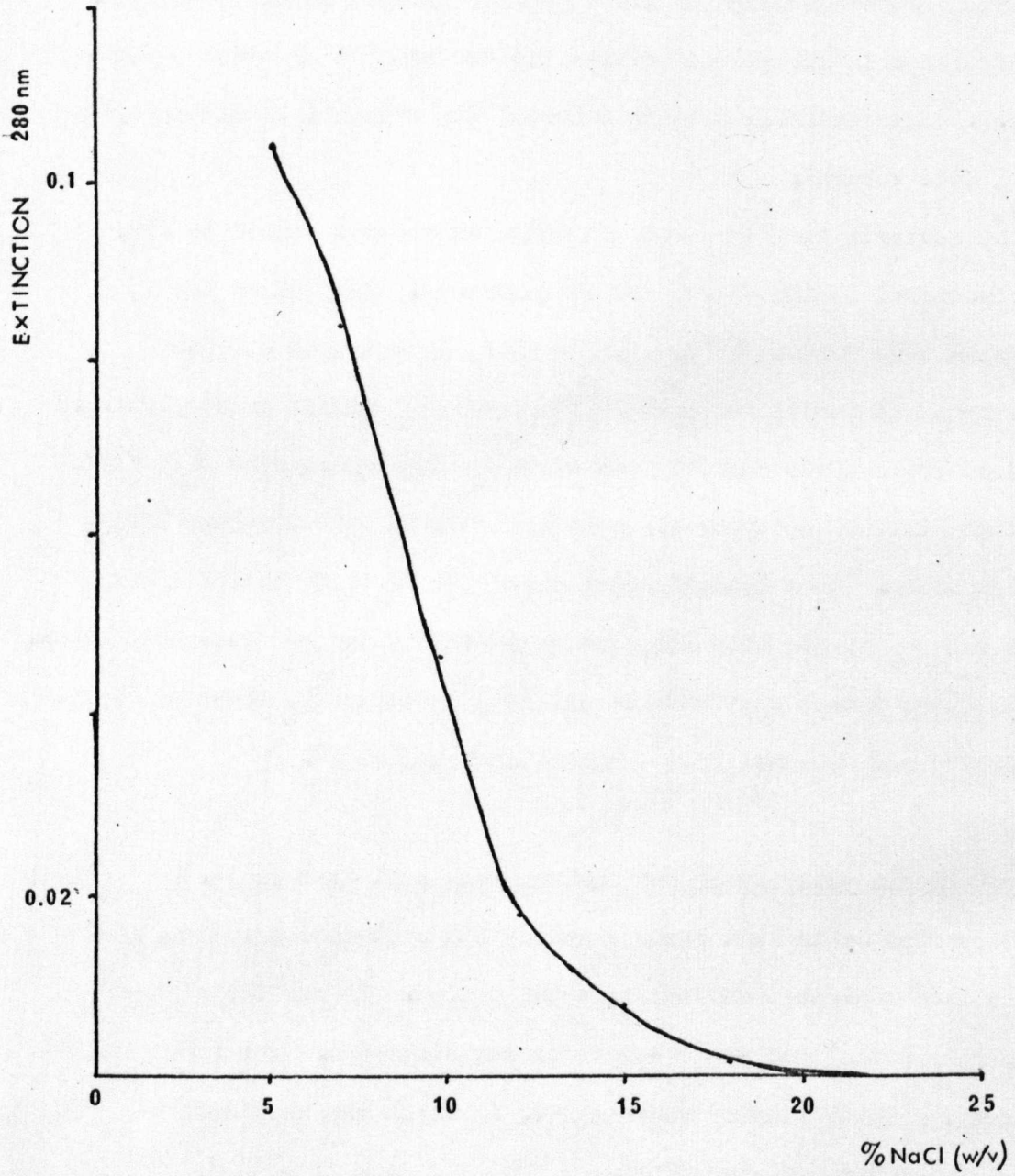

Fig.5. Loss of proteolytic activity with increasing salt concentration.

Bacteria that had been separated from the growth medium were sonicated, and this material was centrifuged. The supernatant was assayed for proteolytic activity with various concentrations of NaCl in the assaying medium.

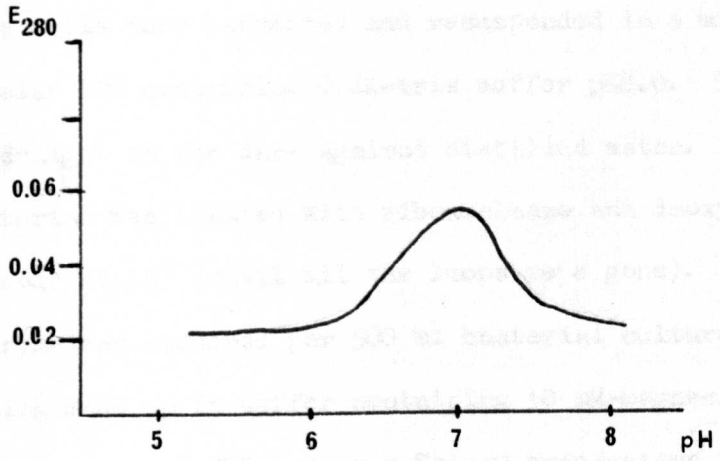

Fig.4. The effect of pH on intracellular proteases.

The assay showed a definite pH effect, with the optimal pH at 7.0.

The dependence of the proteolytic activity of the supernatant on NaCl was investigated. Activity assays were carried out at various salt concentrations. The graph of loss of activity with increasing salt (fig.5.) shows that salt concentrations approaching the extracellular concentration inhibit the enzyme activity. Substitution of KCl for NaCl did not affect the pattern of activity. The effect of the Ca^{2+} ions present in the assay from the casein solution was tested but the presence of the ions was found not to affect the enzyme activity.

The effect of dialysis on the sonicated cell's enzyme activity was investigated. The activity was measured after sonication and the supernatant was dialysed overnight against distilled water at 4^{o}. Then the activity was measured again. As a result of several of these experiments it was found that after dialysis the enzyme activity was enhanced between 3 and 9 times. Dialysis against EDTA only affected the activity to a very slight degree. Addition of NaCl back to the dialysed material brought about almost total inhibition of proteolytic activity.

The proteolytic activity of the cell membranes of H.salinarium.

The cell membranes were prepared after the method of Brown (1969). The cells were harvested and resuspended in a minimum volume of Analar IDM containing 0.1M-tris buffer pH8.0. This was dialysed overnight in the dark against distilled water. The non-diffusible material was treated with ribonuclease and deoxyribonuclease for 1 hour at 35° (until all the lumps were gone). Approximately 2 mg of each enzyme was required per 500 ml bacterial culture originally, the enzymes being made up in buffer containing 10 mM-magnesium ions. The material was spun at 48,000 rpm in a Spinco preparative ultracentrifuge, for 3 h at 0°C. The supernatant was removed and the pellet resuspended (less residue) in distilled water. (Further treatment with the nuclease enzymes was carried out if necessary at this stage.) The resuspended pellet of cell membranes was tested for proteolytic activity with different concentrations of NaCl present. The results are shown in fig.6. It can be seen that the activity was inhibited by the presence of NaCl.

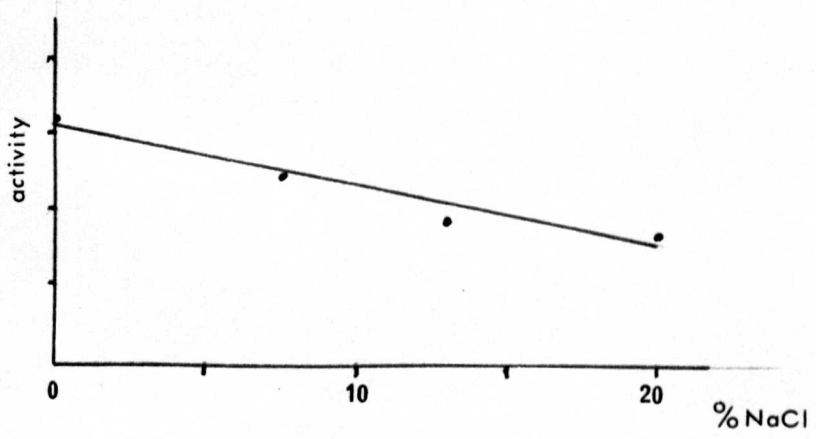

Fig.6. Proteolytic activity of the cell membranes.

Discussion

Proteolytic activity has been demonstrated in three fractions of the H.salinarium bacterial cell. The strain of bacterium used did produce a true extracellular protease, but as pointed out by Norberg and Hofsten(1969)

Halobacterium salinarium 1 does not show very high activity. The retardation of the enzyme on Sephadex G-50 indicated that it had a low molecular weight and could enter into the molecular sieve provided by the Sephadex.

Proteolytic activity was also found to be associated with the cell membranes and with the supernatant from sonicated cells. It may be that the same cell-bound enzyme was involved in each case. In their paper Norberg and Hofsten have identified activity with a cell-bound fraction which they think are cell envelope particles. This would correlate with the activity found to be associated with the membranes and that with the sonicated cells. Both were found to be inhibited by NaCl, and maximum activity was obtained in the absence of salt. Since this enzyme activity was intracellular it is surprising that the enzyme did not show a requirement for K^+ ions. Christian and Waltho (1962) showed that H. salinarium has a very high internal concentration of potassium. Norberg and Hofsten have found that their intracellular proteases require 25% NaCl (w/v) for maximum activity, and they were rapidly and irreversibly denatured in the absence of large concentrations of NaCl. In the case of the enzymes found here, the results of dialysis against distilled water substantiate the suggestion that the enzymes are halophobic. The intracellular enzymes do not appear to require metal ions for activity as dialysis against EDTA had no effect. Norberg and Hofsten claimed that their cell-bound proteinase required Zn^{2+} or Mn^{2+} ions for reactivation after dialysis against EDTA.

It seems therefore, that an extracellular protease has been identified for H. salinarium 1 as described by Norberg and Hofsten. In addition proteolytic activity has been found associated with the cell membranes, and the intracellular material which may be one and the same enzyme. This activity was halophobic as distinct from that of the

the extracellular enzyme which was extremely halophilic as expected.

The physiological function of this halophobic intracellular enzyme is not clear. It must surely be inhibited under normal conditions within the cell. Larsen (1967) has discussed the loss of shape, and sudden lysis of the cells as the NaCl concentration dropped to 5-10%. After lysis the intracellular protease is exposed to much more congenial salt concentrations, and can function as a degradative enzyme bringing about cell membrane digestion.

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and additional explanations made.

After the summary, an index of chapters was added.

p.13 Table 2.2. This was prepared again with more detail of the purification procedure.

p.14 Table 2.3. This was redrawn with more detail of the experimental conditions used.

p.16 The discussion was rewritten to incorporate an account of the methods used to assess enzyme purity, and how it was carried out in this case.

p.19 Fig.3.2. Addition of labels and excitation/emission wavelengths for the fluorescence used.

p.24 Line 17. Addition of experimental details relating to the buffers used.

p.28 Fig.4.3. Legend added.

p.28 Line 13. Additional explanation relating to the buffer used , and its affect on the copper ions present.

p.35 Fig.5.1. Legend added.

p.37 Fig.5.3. Legend added.

p.38 Fig.5.5. Legend added.

p.44 Fig.6.1. Legend added.

p.45 Fig.6.2. Legend added.

p.51 Figs.7.1. & 7.2.: Figs.7.5. & 7.6. Legend added.

p.52 Line 2 Additional explanation added concerning the light intensity used.

p.55 Line 6 Additional explanation regarding the binding of Rose Bengal to a protein.

p.60 Fig.8.1. Legend added.

p.61 Fig.8.2. Points filled in on the figure.

p.64 Line 22. Rate constants corrected.

p.79 Additional explanation about measuring concentration of oxygen for $1/O_2$ plots.

p.81 Line 5. Additional explanation.

Summary of second part of thesis amended.

p.87 Figs.1.,2,3, Legend added to each figure.

p.88 Fig.5. Legend added.